Phenotypic Plasticity in *Rana temporaria* Tadpoles: Lethal and Non Lethal Effects of Multiple Predators

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CONTENTS

GENERAL INTRODUCTION

All organisms exist in variable environments. Such variation represents a critical challenge for the individuals because their performance and fitness can change as the environmental conditions change. Environmental heterogeneity affects the organism in two ways. First, a variable environmental factor influences development, inducing the production of alternative phenotypes as different environmental conditions appear, and second, the environmental factor may have a differential effect on individual fitness and select for phenotypes that have the highest fitness (Moran 1992). Thus, as environmental conditions increase in complexity, a novel multitude of interactions can contribute to produce alternative selective pressures for the organisms (DeWitt and Langerhans 2003). Therefore, our understanding on how the environmental complexity affects the organisms is limited if we focus only on discrete and simple environments.

One of the most important factors structuring communities is predation. Despite the theoretical importance of multi-predator systems and their predominance in nature (Barbosa and Castellanos 2005, Sih et al. 1998), predation has been studied empirically using oversimplified models. Most empirical studies about the predator-prey interactions have examined the effects on prey by using one predator at a time (Relyea 2003). However, as predation varies in composition and abundance, the interactions among predators can

increase and alternative effects on prey can appear (Lima 2002). Therefore, to increase our knowledge about predator – prey interactions it is necessary to complement traditional empirical designs with predator combined conditions, in which the relative proportion of each predator in the combination vary. In this way, new inferences about the effects of dynamic change in both the predator diversity and the predator density can be made, giving us new insights about the complexity in multiple predator systems and allowing us to make a step forward in understanding the ecological function of predation on community structure.

Variation in predator diversity and density represents a challenge for the prey. In animals a series of behavioural, morphological and life history responses have been observed in prey as a response to variation in the risk (Tollrian and Harvell 1999). Such responses improve prey performance and fitness (Moran 1992). However, the effect varies with predator diversity and the effect of the variation in predator density. For example, different responses to separated predators observed in tadpoles, suggest that they can discriminate among predators (Van Buskirk 2001, Relyea 2003). But, on the other side, a fine responses to gradual changes in cue signals emitted by the predators has been documented, suggesting that prey can quantify the predator density (Van Buskirk and Arioli 2002, Schoeppner and Relyea 2008, 2009). These results suggest that in combined multiple predator conditions prey may be able to adjust their responses according to the relative density of each predator in the combination. This means that prey have developed qualitative and quantitative sensitivity to

detect changes in predator cues and have evolved decision rules to adjust their phenotypic responses to simple and complex conditions (Blumstein and Bouskila 1996).

On the other side, variation in the composition and abundance of the predator assemblages can produce changes in prey mortality rate that cannot be predicted from the aggregated effects observed in single and stable predator conditions. For example emergent impacts on prey survival can occur when prey are exposed to combined predator conditions (Sih et al. 1998). This phenomenon has been observed in many multiple predator systems in which the predators produce non additive effects when they are in combination (Schmitz 2007). However, the usual empirical approaches to study this kind of effect, the additive design and the replacement series design, cannot disentangle clearly whether the effects arise by changes in predator diversity or by changes in the total predator density. Thus, to understand the complexity of predation in multiple predator systems and identify emergent multiple predator effects, we require new empirical approaches to identify simultaneously how the change in density at intra- and inter-specific level and how the change in the predator diversity affect prey survival.

Approach to my research questions

Amphibians represent a good model to study the effects of multiple predators. First, they exhibit a notorious variety of predator induced responses, showing changes in behaviour, morphology and life history traits when they are exposed to predation. Second, it is easy to manipulate them during their larval development. Finally, they are very responsive to the presence of water-borne chemical cues emitted by predators. In this study the multiple predator – prey system, was consisted of the *Rana temporaria* tadpoles and the common co-occurring predators: the backswimmer insect *Notonecta glauca*, the adult newts *Triturus alpestris*, and the odonate dragonfly larvae *Anax imperator*. The three predators have different predatory strategies, and in isolation they produce different mortality rates (Van Buskirk and Arioli 2005) and induce different phenotypic responses in tadpoles (Van Buskirk 2001).

I took advantage of this multiple predator system to evaluate how tadpoles responded to different predator conditions and whether these predators produced non additive lethal effects on them. In chapters 1 and 2 I conducted experiments rearing tadpoles in artificial ponds, exposing them to different environmental conditions signaled by the addition of predator kairomones. The kairomones were added to the artificial ponds, manipulating the amount in the pure conditions and the relative proportion in the mixed combined conditions. I also had a predator-free control in which I added aged tap water. To evaluate prey responses, I

measured behavioural, morphological and life history traits at different stages of tadpole development. Using measures obtained in these experiments, I evaluated the dosage response produced by the different predators (chapter 1) and how tadpoles responded to variation in the relative proportion of kairomones when predators occurred in combination (chapter 2). In chapter 3, I exposed tadpoles to conditions in which the predator species and the number of individual predators foraging together varied. Measuring tadpole survival, I determined whether the increase in intraspecific density caused non-additive effects and whether there were emergent multiple predator effects on the tadpoles.

The conclusion obtained in the three chapters extend the results of previous studies in phenotypic plasticity and multiple predator effects, by illustrating how tadpoles perceive the risk, how their decision rules produce phenotypic responses in novel empirical conditions not evaluated before and how the predator combinations impact directly on mortality rate of the prey. Thus, the results will give new insights in the role of predator diversity and trophic interactions in the functioning of ecosystems and encourage ecologist to improve the models of population dynamics and community structure.

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SUMMARY

Predation is one of the most important factors influencing prey population dynamics and community structure. In natural conditions, multiple predator species typically occur together. When these assemblages are composed of species that exhibit different predatory tactics and may represent different levels of threats, first, prey should have specific responses to each predator, and second, they should adjust their response according to the overall risk produced by the predators in combination. Moreover, predator assemblages can change through time in both composition and abundance, so prey should be highly sensitive to variation in predator density and predator diversity. Thus, in single predator conditions it is expected that prey produce specific dosage response curves to predators with different predatory tactics, and in multiple predator conditions adjust their responses according to the relative density of the predators in the combined conditions. On the other hand, the direct effect of predators on prey survival can depend on predator diversity and predator density. When multiple predators have independent effects on prey survival, the effects in more complex predator conditions (such as increasing predator density or total predator diversity) can be predicted from the single predation conditions. However, interactions among predators at intra- and inter-specific level can cause non-additive multiple predator effects that result in increase or reduction of the net mortality rate of the prey. Therefore, the predator species abundance and

composition are important elements that must be incorporated into ecological models that evaluate predator-prey interactions. We now have much information on how prey respond to different predators in separated conditions and how they produce dosage response to a specific predator. Further advance in the functional ecology of predator-prey interactions requires studies of the effects of the dynamic change of predator diversity and predator density.

To fill this gap, first, in a common garden experiment, using artificial ponds, I reared tadpoles of the *Rana temporaria* frog under different levels of apparent predation risk, created by addition of predator kairomones that varied in type and concentration. The kairomones were obtained from three locally-common predators: *Anax imperator* larvae, *Triturus alpestris* adults and *Notonecta glauca* adults. To evaluate tadpole responses, I measured a total of 18 phenotypic responses to variation in apparent risk. These included behaviour, morphology, and performance measures at metamorphosis. Hiding and activity were measured on three occasions during development. Morphology and tadpole mass were measured on two occasions during development, in which I used geometric morphometric methods to describe variation in tadpole shape and centroid size. At tail resorption (stage 45; Gosner 1960) I recorded the time to metamorphosis and measured the metamorph morphology and mass.

In **Chapter 1** I used the responses obtained for each trait from the different pure kairomone conditions of each predator species, which ranged from

250 to 1000 ml in steps of 250 ml, and the responses to the predator-free control treatment to evaluate dosage-response curves for different predators. To identify the type of dosage response to the increase of the amount of kairomone and to determine at trait level whether the dosage response produced by each predator were different, I used model selection based on the Akaike information criteria for small samples (AICc). The models were continuous, threshold and intercept (null) models. The results suggested that tadpoles produce different dosage response curves to different predators, showing both discontinuous and continuous response modes. In broad sense, these results illustrate that the increase of kairomone dose of the three predators caused reduction in tadpole activity, visibility and mass, increase in metamorph mass and time to metamorphosis, and with the exception of some cases in which tadpoles did not respond, caused changes in the shape of tadpoles and metamorphs. Because in most of the cases the response mode depended on the type of predator kairomone added and in few cases the intercept model was identified, the results of this chapter suggest that tadpoles can discriminate quantitatively and qualitatively the predators through kairomone assessment.

In **chapter 2**, for the three pairwise combinations obtained from the three predator species, I evaluated how tadpoles responded to variation in the relative amounts of kairomones in pairwise combinations of predator species. For each pairwise combination, I used the responses obtained in the pure kairomone treatments in high dose (1000 ml), in the predator-free control treatment and in the mixed kairomone treatments in which the relative proportion of kairomones in

the combination were in the ratios 3:1, 2:2, and 1:3. Using planned contrast analyses, I identified the cases in which a generic response was used for all the predator conditions and those in which the predator conditions produced different responses. For the last cases, I used model selection to identify the type of yield response to the kairomone replacement series in combination. I observed that tadpoles exhibit reduction in visibility and activity to all the predation conditions, but in contrast, they did not always produce morphological responses. There were cases in which a single response was used for all the predator conditions and there were cases in which non-lineal and linear yield responses were observed. These results show that the effect of the change in the relative amount of predator kairomone on the tadpole responses is reflected in different ways for different traits, indicating specific-trait decision-making when prey are exposed to combined predators. The decision rules used by tadpoles to respond to variation in predation risk changed over the ontogeny. These responses suggest that the size refuge protection and hunting mode and habitat spatial use of the predators are important elements in the decision making to produce the response.

In **Chapter 3** I evaluated the impacts of multiple predator species on the survival of *Rana temporaria* tadpoles by exposing prey to single predator species with one and two predator individuals and to pairwise combinations of predators. The experiment included three pairwise combinations of *Anax imperator*, *Triturus alpestris* and *Notonecta glauca* predators. I conducted analyses for each pairwise predator system on tadpole survival measured in predation experimental trials that fulfill a complementary design for pairwise predators that merges the

two-by-two factorial design and the replacement series design. First, I determined whether tadpole survival had a lineal relationship with the number of conspecific predators. Second, I determined whether the impact of the combined conditions can be predicted from the impact of each predator in isolation under the assumption of predator species independent effects. This chapter demonstrated that the three predators do not produce emergent effects on tadpole survival when they occur in pairwise combinations. The results also indicate that increasing density of *Triturus* and *Notonecta* does not produce unexpected effects on survival, but in contrast, when the density of *Anax* increases the observed mortality was lower than that expected.

In conclusion, my multi-trait approach extends earlier work by illustrating that tadpoles distinguish qualitative and quantitatively the environmental risk. Also, my results show that the effect of the change in the relative amount of predator kairomone on the tadpole responses is reflected in different ways for different traits, indicating specific-trait decision-making. These results also suggest that tadpoles can use kairomones to estimate the relative abundance of predators in the environment and use this information to adjust their response. The three predator species can be treated as single independent functional units in models that evaluate the prey population dynamics in multiple predators systems.

ZUSAMMENFASSUNG

Prädation ist einer der bedeutendsten Faktoren, der sich auf die Dynamik und Struktur der Beutepopulationen auswirkt. Unter natürlichen Bedingungen sind Lebensräume normalerweise von mehr als nur einer Art von Räubern besiedelt, die sich in ihrer Jagdtaktik und ihrem Grad an Gefährdung für die Beutetiere unterscheiden. Die Beutetiere sollten daher (1) räuberpezifische Reaktionen zeigen, und (2) ihre Reaktion an das Gesamtrisiko, das von der Menge der Räuber ausgeht, anpassen können. Räubergesellschaften können sich zudem sowohl in ihrer Zusammensetzung und Dichte zeitlich verändern, und Beutetiere sollten daher extrem sensibel auf Veränderung in Räuberdichten- und zusammensetzungen reagieren können. Daher geht man davon aus, dass Beutetiere auf die Gegenwart einer einzigen Räuberart mit spezifisch auf deren Taktik abgestimmten Reaktionskurven antworten. Diese Reaktionskurven sollten zudem auf die Gegenwart und relative Dichte mehrerer verschiedener Räuberarten anpassbar sein. Andererseits hängt die direkte Wirkung der Räuber auf das Überleben der Beutetiere von der Räuberdichte- und zusammensetzung ab. Wenn mehrere verschiedene Prädatoren unabhängig voneinander das Überleben der Beutetiere beeinflussen, können die Auswirkungen von komplexeren Räuberzusammenstellungen (zum Beispiel erhöhte Räuberdichten oder -diversität) ausgehend von den Auswirkungen der Gegenwart einzelner Räuber vorhergesagt werden. Intra- und interspezifische Interaktionen hingegen

können zu nichtadditiven Effekten führen, die ihrerseits einen Anstieg oder Abfall der Gesamtmortalität der Beutetiere bewirken können. Die Dichte und Zusammensetzung der Räuberarten sind daher wichtige Faktoren in ökologischen Modellen, die die Interaktionen zwischen Räuber und Beute abschätzen. Heute verfügen wir über eine Vielzahl an Informationen darüber, wie und mit welchen spezifischen Reaktionskurven Beutetiere auf verschiedene, getrennt präsentierte Prädatoren in unterschiedlichen Dichten reagieren. Um mehr über die funktionelle Ökologie von Räuber-Beute-Beziehungen zu erfahren, benötigt es Studien über die Auswirkungen dynamischer Veränderungen von Prädatorendiversität und –dichte.

Um diese Kenntnislücke zu schliessen, habe ich ein ‚common garden' Experiment mit *Rana temporaria* Kaulquappen durchgeführt: Ich zog die Kaulquappen unter verschiedenen Bedingungen auf, die sich in Art und Konzentration verschiedenen Prädationsrisiken unterschieden. Das Vorkommen der Räuber simulierte ich durch variierende Zugabe von Räuberkairomonen. Die Kairomone wurden von drei lokal häufig vorkommenden Räubern bezogen: *Anax imperator* im Larvalstadium, *Triturus alpestris* im Adultstadium, und adulte *Notonecta glauca*. Um die Reaktion der Kaulquappen auf das variierende Prädationsrisiko zu evaluieren, habe ich insgesamt 18 phänotypische Reaktionen gemessen, darunter das Verhalten, die Morphologie sowie Merkmale, die in Zusammenhang mit Fitness nach der Metamorphose stehen. Der Aktivitätslevel wurde dreimal während der Entwicklung der Kaulquappen ermittelt, während

Morphologiedaten und Körpergewicht zweimal während der Entwicklung erhoben wurden. Geometrisch-morphometrische Methoden wurden hinzugezogen, um Variation in Körperform und Centroidgrösse abzuschätzen. Nach Resorption der Schwanzflosse (Stadium 45; Gosner 1960) wurden die Zeit, die jede individuelle Kaulquappe bis zur Metamorphose benötigte und das erreichte Körpergewicht registriert, und die Körperform wurde vermessen.

Im **1. Kapitel** habe ich die prädatorspezifischen Reaktionskurven aller an den Kaulquappen gemessenen Merkmale bestimmt, indem ich die Reaktionen jedes einzelnen Merkmales auf die verschiedenen reinen Kairomone der einzelnen Räuber mass. Dabei setzte ich die Kaulquappen Kairomonen des Prädators in unterschiedilchen Dosen aus: 0 (Kontrolle), 250, 500, 750 und 1000 mL. Um die Art der Reaktion auf einen Kairomonanstieg zu identifizieren und zu bestimmen, ob sich die Reaktionen auf verschiedene Räuberarten voneinander unterscheiden, habe ich Modelle basierend auf dem Akaike Informationskriterium für kleine Stichproben (AICc) selektiert. Dabei verglich ich kontinuierliche, Grenzwert- und Intercept- (Null-) Modelle. Die Resultate zeigen, dass Kaulquappen auf die Gegenwart verschiedener Prädatoren mit unterschiedlichen Reaktionskurven reagieren, die sowohl kontinuierlich als auch diskontinuierlich sein können. Insgesamt illustrieren meine Resultate, dass ein Anstieg an Kairomonen der drei oben erwähnten Räuberarten bei den Kaulquappen zu einer Reduktion ihrer Aktivität und ihres Körpergewichtes führt; der Zeitraum, den eine Kaulquappe bis zum Erreichen der Metamorphose braucht, wird verlängert und

das Körpergewicht nach Abschluss der Metamorphose erhöht. Abgesehen von einigen Ausnahmen passten die Kaulquappen und frisch metamorphosierten Jungtiere auch ihre Morphologie an. Da in den meisten Fällen die Art der Antwort räuberspezifisch war, schnitt in nur wenigen Fällen das Nullmodell am besten ab. Dies deutet darauf hin, dass Kaulquappen dazu in der Lage sind, Prädatoren aufgrund ihrer Kairomone sowohl quantitativ als auch qualitativ zu unterscheiden.

Im **2. Kapitel** habe ich getestet, wie Kaulquappen auf die Variation der relativen Konzentrationen an Kairomonanteilen verschiedener Räuberarten reagieren. Dazu benützte ich paarweise Kairomonkombinationen der drei bereits erwähnten Räuberarten. Ich setzte die Kaulquappen folgenden Bedingungen aus: eine Kairomon-freie Kontrolle, gemischte Kairomone im Verhältnis 3:1, 2:2 und 3:1 mit einer Endkonzentration von 1000 mL, und reine Kairomone der Prädatoren bei einer Konzentration von 1000 mL. Mithilfe von Kontrastanalysen habe ich die Fälle identifiziert, in denen bei allen Behandlungen eine allgemeine unspezifische bzw. eine spezifische Antwort hervorgerufen wurde. Für die spezifischen Reaktionen benützte ich Modellselektion, um die Art der Reaktion auf die relativen Anteile an Kairomonen der verschiedenen Prädatoren zu bestimmen. Die Kaulquappen reagierten auf die Gegenwart von Fressfeinden immer mit einer Reduktion ihrer Aktivität und Sichtbarkeit, veränderten allerdings nur in bestimmten Fällen ihre Morphologie. Während manche Reaktionen gleich stark für alle Bedingungen ausfielen, wurden andere in ihrer Ausprägung sowohl

in linearer als auch in nicht-linearer Weise angepasst. Diese Resultate legen dar, dass der relative Anteil an Räuberkairomonen sehr unterschiedliche Wirkungen auf die Antwort der Kaulquappen haben kann und sich je nach Merkmal unterscheiden. Dies weist darauf hin, dass Beutetiere merkmalsspezifische Entscheidungen treffen können, wenn sie einer Kombination mehrer Fressfeine gegenüberstehen. Diese Entscheidungsregeln verändern sich während der Ontogenie der Tiere. Räuberart, räumliche Verteilung der Räuber und ab wann die Beute durch ihre Grösse vor dem Räuber geschützt ist, sind daher wichtige Elemente für die Entscheidungsregeln der Reaktionskurven.

Das **3. Kapitel** geht auf die Frage ein, welche Auswirkung die Anwesenheit mehrerer Prädatorenarten auf das Überleben von *Rana temporaria* Kaulquappen haben: die Beutetiere wurden einzeln einem oder zwei Räubern präsentiert, die entweder Vertreter derselben Art oder zweier verschiedener Arten waren. Im Experiment wurden die folgenden Prädatoren paarweise kombiniert: *Anax imperator*, *Triturus alpestris* und *Notonecta glauca*. Für jede paarweise Kombination analysierte ich, welche Wirkung die Fressfeinde auf das Überleben der Kaulquappen in jedem experimentellen Durchlauf hatten. Die Versuche wurden nach einem komplementären Design für paarweise Prädatoren durchgeführt, einer Kombination eines ’two-by-two’ faktoriellen und eines ’replacement series’ Designs. Zuerst habe ich untersucht, ob das Überleben der Kaulquappen in einer linearen Beziehung zur Anzahl der Fressfeinde gleicher Art steht. Zweitens habe ich bestimmt, ob die Wirkung, die von der Kombinationen

von Fressfeinden unterschiedlicher Art ausgeht, aufgrund der Wirkung isolierter Prädatoren vorhergesagt werden kann, wenn man davon ausgeht, dass Fressfeinde verschiedener Arten unabhängige Effekte zeigen. Dieses Kapitel hat gezeigt, dass die drei in meinem Experiment eingesetzten Fressfeinde keine erhöhte Auswirkung auf das Überleben der Kaulquappen haben, wenn sie paarweise kombiniert werden. Die Ergebnisse weisen darauf hin, dass ein Anstieg der Dichte von *Triturus* und *Notonecta* das Überleben der Kaulquappen nicht unerwartet stark reduziert, während ein Anstieg in der Dichte von *Anax* die Überlebensrate sogar weniger stark verringert als erwartet.

Zusammenfassend lässt sich sagen, dass mein Ansatz, eine Vielzahl an Merkmalen zu untersuchen, frühere Arbeiten auf diesem Gebiet erweitert hat. Ich konnte zeigen, dass Kaulquappen die Risiken, die von ihrer Umwelt ausgehen, sowohl qualitativ als auch quantitativ unterscheiden können. Meine Resultate belegen ausserdem, dass eine Veränderung in der relativen Konzentration an Kairomonen unterschiedlicher Räuber verschiedenartige Veränderungen in den untersuchten Merkmalen der Kaulquappen bewirkt. Dies deutet auf merkmalsspezifische Entscheidungsregeln hin. Kaulquappen sind ausserdem dazu in der Lage, anhand der Kairomonkonzentrationen in ihrer Umgebung die relative Häufigkeit verschiedener Räuber abschätzen zu können, und diese Information in ihrer Antwort auf die Gegenwart von Fressfeinden zu nutzen. Die in meinem Experiment eingesetzten drei Arten von Fressfeinden können als einzelne, unabhängige funktionelle Einheiten behandelt werden, wenn sie in

Modelle eingefügt werden, um Dynamiken von Beutetierpopulationen in Systemen mit mehreren Prädatoren abzuschätzen.

CHAPTER 1

DOSAGE RESPONSES TO DIFFERENT PREDATOR SPECIES IN *RANA TEMPORARIA* TADPOLES: EFFECTS OF VARIATION IN PREDATOR KAIROMONE COMPOSITION

Oscar Javier Ramos Real

Abstract

Predator-induced plasticity has been used widely to assess how organisms adjust their phenotypes in response to changes in their environment. In a multiple predator context, specific induced responses to the presence of different predator species have been well documented. However much less has been done to evaluate whether prey adjust their responses according to the probability of specific encounters. For three different predator species, I examined dosage response curves of tadpoles, in which the increase of risk was signaled by addition of predator kairomones that varied in concentration. This study demonstrates that the dosage response curves depended on the trait and the predator species. Continuous and threshold response curves described reduction in tadpole behaviour, reduction in tadpole mass, increase in time to metamorphosis and changes in shape morphology at metamorph stage, whereas the increase in shape morphology at larval stage and mass at metamorphosis were described only by threshold response curves. The results imply that chemical cues are enough to trigger the full range of phenotypic responses in tadpoles, indicating that tadpoles have qualitative and quantitative sensory sensitivity to evaluate the risk caused by different predator species, and suggest that tadpoles apply assessment rules to the kairomones information to produce specific phenotypes.

Introduction

In natural communities, prey usually face fluctuations in predation risk. In such variable conditions prey have evolved predator-induced plasticity in defensive traits (Tollrian and Harvell 1999). However, when prey face multiple predator species that exhibit different predatory tactics and that may represent different levels of threats, they should adjust their response according to the probability of specific encounters (Richardson 2001, Altwegg 2003, Teplitsky et al. 2005) and to the associated costs of production and maintenance (Moran 1992, Tollrian and Harvell 1999). Under these conditions, prey must estimate the predator species abundance and composition to produce the appropriate response. Indirect cues of predator presence are an important source of information about the environmental risk (Lima and Steury 2005). It is expected that prey exposed to complex conditions have evolved qualitative and quantitative sensitivity to detect changes in predator cues.

In aquatic animals, a broad array of phenotypic responses to predation have been identified, induced by chemical cues diluted in the water (Tollrian and Harvell 1999, Laforsch and Tollrian 2004, Richardson 2006). A number of experiments have focused on the qualitative prey sensitivity to different predator species, while others, using only one predator species, have focused on their quantitative sensitivity. For amphibians, predator-specific responses using presence/absence conditions for separate caged predator species suggest that

tadpoles can discriminate among different kairomone types (Van Buskirk 2001, Relyea 2001, Storfer and White 2004, Teplitsky et al. 2004). On the other hand, manipulation of predator density and diet of a defined predator species, signaling mono-specific gradual increase of risk, produce dosage-response curves in tadpole behaviour and morphological traits (Van Buskirk and Arioli 2002, Schoeppner and Relyea 2008, 2009). Although these studies suggest that tadpoles exhibit qualitative and quantitative adaptive sensitivity to predator cues, they represent an oversimplification of the conditions under which prey encounter predators. More realistic experiments manipulating both amount and kind of predator cues are necessary to determine how prey specifically respond to the variation of density of different predator species.

It is impossible to know what prey perceive about predation risk (Blumstein and Bouskila 1996). However, prey responses obtained from manipulation of different kind of predator support indirect inferences about prey sensitivity (Lima and Steury 2005). As the adaptive sensitivity to predator cues is one of the foundations for the evolution of inducible defenses (Moran 1992, Tollrian and Harvell 1999), dosage response curves to the variation in the amount of cue can represent how accurate the prey perceive risk. Further, differences among dosage curves for different predators that cause different survival effects on prey would suggest that the perception is fine tuning according with the specific risk.

Anax imperator larvae, *Notonecta glauca* adults and *Triturus alpestris* adults are aquatic predators that frequently co-occur with *Rana temporaria* tadpoles. They cause different mortality on tadpoles (Van Buskirk and Arioli 2005) and trigger different tadpole responses in behaviour (Van Buskirk 2001). As these predator species represent different threats for tadpoles and specifically tadpoles of *R. temporaria* can discriminate them, it is expected that they can as well differentiate the quantitative variation of their cues. Therefore, here I plan to characterize the dosage responses curves of tadpole behaviour, morphology and life history in *Rana temporaria* exposed to different amounts of kairomones coming from these three different predators. I use this as an indirect way to evaluate whether tadpoles have developed qualitative and quantitative sensitive abilities to judge changes in the environmental risk.

Methods

In a common garden experiment using artificial ponds, I reared tadpoles of the frog *Rana temporaria* under different levels of apparent predation risk created by addition of predator kairomones that varied in type and concentration. To evaluate the tadpole responses, I measured behaviour on three occasions, tadpole morphology on two occasions, and performance measures at metamorphosis. The kairomones were obtained from the locally-common dragonfly larvae *Anax imperator*, adult newts *Triturus alpestris*, and adult backswimmers *Notonecta glauca*.

The experimental units were plastic tubs (0.28 m^2, 80 L) placed outdoors in a field at the University of Zurich, Switzerland. On 10 – 11 March 2008, two weeks before the experiment started, I filled the tubs with tap water and added to each 50 g of leaf litter, 2 g of rabbit food and, on two occasions, two aliquots of diluted zooplankton to simulate natural conditions and establish mesocosms for the tadpoles. I covered the tubs with cloth lids to prevent colonization by aquatic predators or other amphibians.

The experimental design consisted of 13 predation environments created by daily addition of different aliquots of diluted predator kairomone (250, 500, 750 and 1000 ml). For the predator-free environment (control) I added 1000 ml of aged tap water. There were seven replicates of the 12 kairomone treatments and 21 replicates of the predator-free control, resulting in a total of 105 experimental units in a completely randomized design.

The diluted kairomones were produced by 24 individuals of each predator species. I kept predators individually for 24 hours after feeding in 1L plastic boxes. Every predator was fed daily within the box at 8:00 – 9:00 with ~300 mg of *R. temporaria* tadpoles (4-6 tadpoles). At 7:00 – 8:00 on the next day, I collected the water contained in the boxes, refilled them with aged tap water, and fed the predators again. The water containing kairomones was diluted to a total volume of 48L, yielding a concentration of 150 mg-tadpole/L per predator species. The

final concentration in the experimental units was between 0.46 and 1.9 mg-tadpole/L depending on the treatment. I replaced a predator if it consumed less than 70% of its food, and I replaced all predators weekly with new individuals from nearby ponds.

On 23 March, the experiment began when I introduced 14 tadpoles to every mesocosm (hatchlings were three days old, Gosner (1960) stage 23 – 25, 15.7 mg). The tadpoles came from clutches of *Rana temporaria* collected on 13 and 14 March 2008 in seven different ponds, distributed over an area of ~120 Km^2 in northern Switzerland. Kairomone addition continued daily from 24 March until 28 May, by which point 64% of tadpoles had reached metamorphosis. The experiment ended when the last froglet emerged.

Phenotypic responses to predator kairomones

I measured the response to variation in apparent predation risk by sampling behaviour and morphology during the larval period and measuring metamorphic features at stage 45 (tail resorption complete). To evaluate the behavioural responses, I measured activity and visibility when the tadpoles were 19, 36 and 48 days old. The behaviour measurements were collected between 10:00 and 15:00, visiting the mesocosms 6 - 7 times each. In each visit I counted the number of tadpoles that were visible above the leaf litter and the number that were active (swimming, moving the tail, or feeding). The proportion visible was

the number of individuals observed divided by the initial number in the tub (14 tadpoles; survival range at the end of the experiment: 12 to 14 individuals). Activity was the number of active tadpoles divided by the number of visible tadpoles.

I measured the size and shape of five tadpoles from each mesocosm when they were 37-38 and 49-50 days old. Each individual was anesthetized in benzocaine to reduce activity, weighed, and photographed in lateral view within a small water-filled Plexiglas chamber using a digital camera. No mortality occurred by the anesthetic procedure, and tadpoles were returned to their original mesocosm after they recovered. I digitized the coordinates of the 31 landmarks shown in Appendix A using image analysis software (Image J; http://rsbweb.nih.gov/ij/). Geometric morphometric methods were used to describe the variation in tadpole shape and centroid size (CS), which was calculated as the square root of the sum of squared distances of the landmarks from their centroid. Landmarks were scaled by centroid size and aligned using generalized least squares Procrustes superimposition (GLS). Shape components were obtained from a principal component analysis on the partial warps. This procedure produced 58 PCs (relative warps - RW). I retained the first three RWs, accounting for 74.3% of the variance in shape, for later analyses. The morphometric analyses were implemented in IMP software written by H.D. Sheets (http://www3.canisius.edu/~sheets/moremorph.html)

The first tadpoles reached metamorphosis, defined as stage 42, when they were 58 days old. After this date, I checked all the mesocosms every day and transferred individuals in stage 42 to translucent plastic boxes (18 x 12 x 9 cm) containing a small layer of water. For all the individuals I recorded the date of tail resorption (stage 45). For the metamorph morphological measurements, I photographed and weighed a subsample of five haphazardly chosen individuals from each mesocosm at stage 45. I obtained six morphological measurements using Image J: head width, body length (snout-vent length), upper hind leg length, width of the upper hind leg, lower hind leg length and width of the lower hind leg (Appendix B.1). The leg measurements were measured on both sides and the average was taken. After I performed a principal component analysis on these measurements, I retained the two first components, representing metamorph body size (PC1: 84.71% of the variance) and limbs length (PC2: 9.90% of the variance) (Appendix B.2).

Statistical analysis

My analysis was designed to identify how tadpoles respond to the increase of kairomone amount of different predator species rather than whether any particular response was significant. For each trait and each predator, I compared alternative models relating the trait response to the amount of predator kairomone. I used the small-sample version of Akaike Information Criteria (AICc) and model selection (Burnham and Anderson 2002) to identify the best model

supported by the data from a set of four biologically-justified alternative models. Akaike weights (*w*) were used to identify which model support better the tadpole response.

I proposed models that represent typical patterns of phenotypic plasticity in traits with continuous or discontinuous variability (Via et al. 1995, Berrigan and Scheiner 2004, David et al. 2004). Therefore, I described continuous phenotypic variation with linear and exponential models and discontinuous variation with a predator threshold model. Because in the treatments I used four different aliquots of diluted predator kairomone and water addition, for the threshold model, a preliminary selection was performed among four models in which the break point of the step function was located in each of the four aliquot changes. The exponential model was proposed to evaluate whether there is saturation in the sensorial system or during the phenotype production, and the threshold model was proposed to evaluate whether tadpole sensitivity and the phenotypic regulatory machinery requires a minimum signal level to trigger a character state change. I also used a null model, including only the intercept, to identify the case in which tadpoles do not respond to the increase of kairomone dose.

The analyses were performed on residuals after controlling for differences among replicates (ponds of origin), because I was not specifically interested in population differences. Data Processing and model fitting were performed using R version 2.9.1.

Results

Tadpoles showed different response modes to the increase of kairomone amount of the different predators, but always in the same direction. In broad sense the main responses were reduction in activity and visibility, decrease in tadpole mass, and increase in time to metamorphosis and mass at metamorphosis. Morphological shape tended to show discontinuous changes with increasing risk in the tadpole stage, but both continuous and discontinuous responses at the metamorph stage. There were also several traits that did not respond to predators.

The best supported dosage response curves depended on the trait and the predator species (Table 1). Activity and visibility responses were mainly described by the threshold and negative exponential modes, indicating that even low kairomone doses represent high risk for the tadpoles (Fig. 1). *Anax* and *Triturus* nearly always produced continuous responses, showing consistent evidence for an exponential decay in visibility for the first sample and in activity for the second, whereas *Notonecta* with one exception triggered threshold responses (Fig. 1). The shape of the body was best explained by the threshold model or by the model with only an intercept (Table 1). When the shape scores were affected by the kairomone doses, a threshold increase in the score value was observed (Fig. 2). RW2 was the only shape trait that showed response to all predators; *Anax* induced the strongest effect. Further, *Anax* was the only

predator that produced threshold responses in nearly all morphological shape scores. All the predators caused tadpoles to decrease their mass. The best model describing this response was usually the threshold model. Only in the *Anax* case, at day 49-50, the data supported the linear model for this trait (Fig 2). Mass at metamorphosis was best described by the threshold model, in which increase is observed at high kairomone doses. For froglet morphology at metamorphosis, responses were detected only to *Triturus* and *Notonecta*, which were best described by linear and threshold models. The response data of the time to metamorphosis for *Triturus* and *Notonecta* supported continuous models while for *Anax* data supported the threshold model. Regardless of the response mode, the time to metamorphosis increased as the kairomone dose increased (Fig. 3).

Discussion

This study demonstrates that tadpoles produce different kinds of dosage response curves to different predators, showing both discontinuous and continuous response modes. Strong differences in the AIC weights support that different models apply to the different predators, indicating that tadpoles produce specific dosage responses. The increase of kairomone dose of the three predators caused reduction in tadpole activity, visibility and mass, increase in metamorph mass and time to metamorphosis, and with exception of some cases in which tadpoles did not respond, caused changes in shape of tadpoles and

metamorphs. In broad sense, based on the direction of the responses, the results confirm previous studies, which indicate tadpole activity reduction, tail depth increase, tadpole mass decrease, delay in metamorphosis, and increase in mass at metamorphosis when they are exposed to the presence of predators (Skelly and Werner 1990, Skelly 1994, Relyea 2001, Van Buskirk 2002, Teplitsky et al. 2005, Richardson 2006, Urban 2008). But my multi trait approach goes beyond earlier work by illustrating how tadpoles can detect environmental risk qualitatively and quantitatively.

There is no direct way to asses the accuracy of risk perception (Lima and Steury 2005). The knowledge in this area has been inferred from stimuli-response models evaluating non-lethal effects of predators on prey that comprise a series of fundamental stages: perception-filtering of information, the assessment of the filtered information and the decision making process (Blumstein and Bouskila 1996). Under this restrain, wrong conclusions about limitations in the prey perception may be obtained when one or few traits are evaluated. For example, by chance, observations made only on unresponsive traits, when other traits not evaluated respond, prevent us from recognizing that indeed prey can perceive the risk. Thus, under the assumption that the stimulus perception-filtering stage in the sensorial system is common for the suite of traits evaluated in this study, my findings, in which each predator caused dosage continuous response at least in one trait, and in which for some traits the three predators triggered different modes in the dosage response, support the

conclusion that tadpoles are able to discern quantitative and qualitatively the risk imposed by the three predators.

Leaving aside possible limitations in perception of risk, then the differences observed in the dosage-responses must result from the information assessment and decision making processes. Such responses may be adaptive responses that provide specific antipredatory benefits. The main benefits of behavioural antipredatory strategies include reducing predation risk by interrupting the predator cycle at the pre-contact stages (Jeschke 2006). In this study, the behavioural responses were threshold for *Notonecta* and continuous for *Anax* and *Triturus*. Because these predators differ in their habitat distributions and hunting modes, it is possible that the differences in the behavioural responses confer specific spatial avoidance benefits. *Notonecta* is a visual predator that uses the open water column, diving to capture prey from its searching position at the water surface. When tadpoles move out from the shelter of leaf litter to the open water column in this predator condition, the probability of detection is high regardless of the predator density. Therefore, the adaptive tadpole response in this case may be to remain hiding even when a small amount of *Notonecta* cue is detected. On the other hand, *Anax* and *Triturus* search for prey by moving slowly through the vegetation and using sudden attacks at close range. Because the vegetation structure makes visual detection of predators difficult, predation risk depends mainly on the probability of encounter, which is function of predator density. Under these conditions, the

beneficial response for prey may be to reduce activity in accordance with the increase in *Anax* or *Triturus* cues. Responding too strongly, as for *Notonecta*, may generate unnecessarily costs (i.e. reduced foraging opportunities). These arguments would have been better supported if I had observed a greater reduction in tadpole size and mass at metamorphosis in the *Notonecta* treatment than with the other predators. My results do not show such a result, suggesting that tadpoles can maintain similar levels of energy intake regardless of the predator conditions and hiding behaviour.

Tadpole morphological responses to predators differ from behavioral responses in that they function at advanced stages of the predation cycle (Jeschke 2006). It has been shown that responses in the shape of the tail improve survival in presence of predators (Van Buskirk and Relyea 1998, Van Buskirk and McCollum 2000) by increasing the swimming performance, conferring escaping advantages and/or generating distraction to the predator (Van Buskirk and McCollum 2000, Teplitsky et al. 2005). My results show that tadpole morphology responses, when they occurred, were only threshold responses and were more strongly influenced by *Anax*. In fact, once the predation sequence reaches the attack stage, the predator-prey interaction occurs one to one and predator density is not anymore an important factor. However, at that level, the tadpole morphology responses can confer different benefits, according to the tadpole vulnerability to each predator. While *Anax* consumes prey using external mandibles, with options of consumption on a

broad range of tadpole sizes, *Notonecta* and *Triturus* are gape limited and can consume tadpoles only at early stages when the prey are small. This makes tadpoles vulnerable to *Anax* during a longer period of the developmental time than for the other predators. Therefore, tadpoles exposed to *Anax* that invest more in shape, producing of deep muscles and tails, can obtain the benefits of escape or at least reduce the probability of a lethal attack. In this study I observed that tadpole mass was more affected by *Anax* than by other predators, and the time to metamorphosis was longest for tadpoles in *Anax* predation condition. These responses, in contrast to behaviour, may be a consequence of the investment in the tail, suggesting evidence of the potential costs of the investment in shape.

The multi trait approach of my study has established that chemical cues are enough to trigger the full range of phenotypic responses in amphibian larvae. The approach allowed me to observe how tadpoles respond when the type and amount of kairomone change. The main conclusion is that tadpoles have qualitative and quantitative sensory sensitivity to evaluate the risk caused by these three co-occurring predators. Further, it is likely that tadpoles apply assessment rules to the kairomone information to produce specific phenotypes that decrease predation risk by reducing the probability of detection, encounter or even attack. However, this study explored conditions simulating separate single predator situations. Therefore, predictions about responses to multiple predator conditions that can be made from these results are valid only if the predator

species have independent effects (Sih et al. 1998). Because not always the responses to multiple predators combine additively (Relyea 2003), evaluating simultaneously the effects of variation in predator diversity and predator density on prey responses is a necessary next step to understand more about community structure and the effects underlying the inherent complexity of species interactions (DeWitt and Langerhans 2003).

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Tables

Table 1. Akaike weights of the models used for model selection based on the small sample Akaike Information Criteria (AICc) for traits measured on tadpoles of *Rana temporaria* when they were exposed to different amount of kairomones obtained from three different predators: a) *Anax imperator* larvae, b) *Triturus alpestris* adults and c) *Notonecta glauca* adults. The measurements were obtained at different developmental stages, indicated in front of the trait name by the tadpole age (except for traits measured at metamorphosis). K is the number of estimable parameters in the model. The best fitting model is highlighted in boldface. The Akaike weights for all traits always sum to 1. RW are the relative warps representing morphological shape illustrated in figure 2, obtained from the geometric morphometric analysis.

	Model			
Trait	Intercept	threshold	linear	exponential
K	**2**	**3**	**3**	**3**
a) *Anax imperator*				
Visibility - Day 19	0.000	0.125	0.000	**0.875**
Activity - Day 19	0.000	0.102	0.000	**0.898**
Visibility - Day 36	0.000	0.004	0.258	**0.738**
Activity - Day 36	0.000	**0.805**	0.000	0.195
Visibility - Day 48	0.000	0.016	0.004	**0.980**
Activity - Day 48	0.000	0.069	**0.689**	0.242
Tadpole mass - Day 37-38	0.000	**0.634**	0.007	0.359
Tadpole mass - Day 49-50	0.000	0.157	**0.543**	0.300
RW 1 - Day 37-38	0.136	**0.561**	0.129	0.174
RW 1 - Day 49-50	0.058	**0.580**	0.170	0.191
RW 2 - Day 37-38	0.000	**0.526**	0.165	0.309
RW 2 - Day 49-50	0.216	**0.472**	0.239	0.073
RW 3 - Day 37-38	0.001	**0.585**	0.239	0.176
RW 3 - Day 49-50	**0.545**	0.205	0.184	0.066
Mass at metamorphosis	0.078	**0.426**	0.189	0.307
Body size at metam (PC)	**0.415**	0.290	0.188	0.107
Limbs length (PC)	**0.375**	0.334	0.173	0.118
Time to metamorphosis	0.000	**0.760**	0.007	0.234

Table 1. Continued

Trait	Model			
	Intercept	threshold	linear	exponential
K	**2**	**3**	**3**	**3**
b) *Triturus alpestris*				
Visibility - Day 19	0.000	0.002	0.000	**0.998**
Activity - Day 19	0.000	0.001	0.001	**0.998**
Visibility - Day 36	0.000	0.011	**0.536**	0.453
Activity - Day 36	0.000	0.015	0.478	**0.507**
Visibility - Day 48	0.000	**0.765**	0.001	0.234
Activity - Day 48	0.000	0.038	0.198	**0.764**
Tadpole mass - Day 37-38	0.000	**0.395**	0.263	0.341
Tadpole mass - Day 49-50	0.000	**0.704**	0.166	0.130
RW 1 - Day 37-38	**0.407**	0.321	0.208	0.064
RW 1 - Day 49-50	**0.448**	0.283	0.184	0.085
RW 2 - Day 37-38	0.002	**0.599**	0.180	0.219
RW 2 - Day 49-50	**0.564**	0.195	0.181	0.060
RW 3 - Day 37-38	**0.370**	0.300	0.241	0.089
RW 3 - Day 49-50	**0.562**	0.202	0.180	0.055
Mass at metamorphosis	0.070	**0.642**	0.216	0.071
Body size at metam (PC)	0.011	**0.894**	0.036	0.059
Limbs length (PC)	**0.411**	0.291	0.210	0.088
Time to metamorphosis	0.000	0.218	**0.488**	0.293
c) *Notonecta glauca*				
Visibility - Day 19	0.000	**0.766**	0.000	0.234
Activity - Day 19	0.000	**0.666**	0.000	0.334
Visibility - Day 36	0.000	**0.621**	0.000	0.379
Activity - Day 36	0.000	0.339	0.059	**0.602**
Visibility - Day 48	0.000	**0.755**	0.000	0.245
Activity - Day 48	0.000	**0.555**	0.010	0.435
Tadpole mass - Day 37-38	0.000	**0.684**	0.003	0.312
Tadpole mass - Day 49-50	0.000	**0.590**	0.062	0.347
RW 1 - Day 37-38	**0.521**	0.256	0.168	0.055
RW 1 - Day 49-50	0.324	**0.371**	0.224	0.081
RW 2 - Day 37-38	0.013	**0.870**	0.060	0.057
RW 2 - Day 49-50	**0.360**	0.284	0.231	0.126
RW 3 - Day 37-38	**0.486**	0.252	0.200	0.061
RW 3 - Day 49-50	**0.396**	0.326	0.177	0.102
Mass at metamorphosis	0.132	**0.516**	0.270	0.083
Body size at metam (PC)	0.184	0.329	**0.354**	0.132
Limbs length (PC)	0.206	**0.609**	0.125	0.061
Time to metamorphosis	0.000	0.365	0.046	**0.588**

Figures

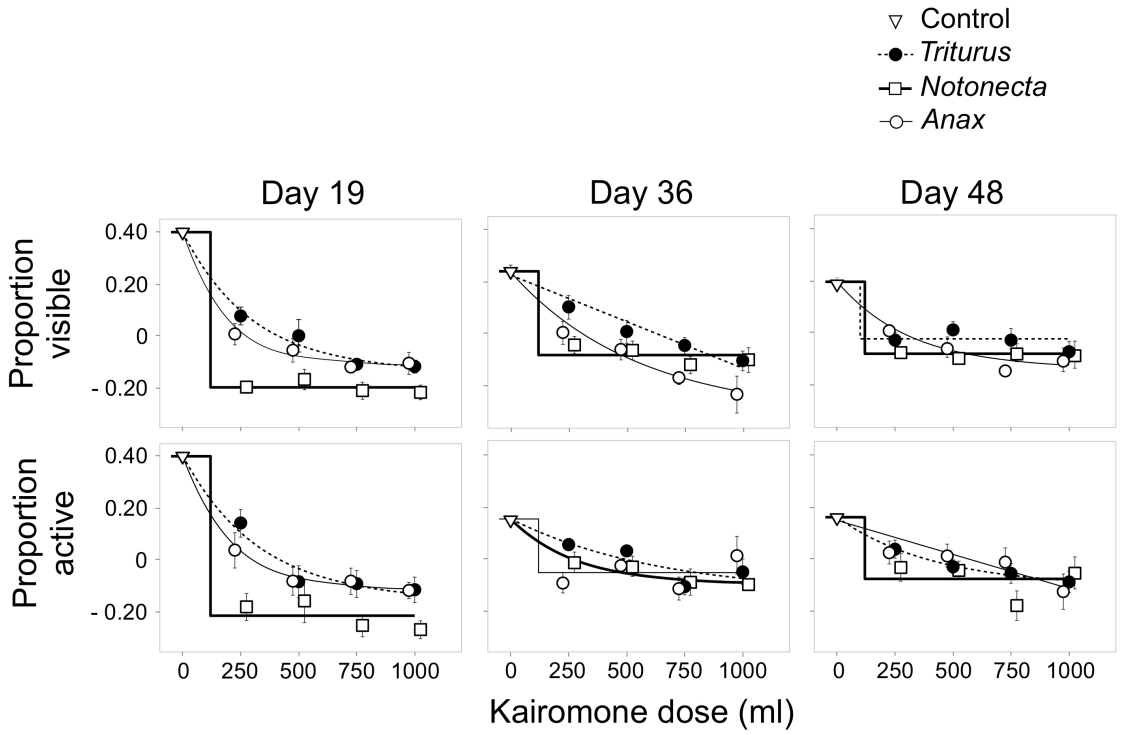

Figure 1. Behavioural responses of *Rana temporaria* tadpoles to the increase of kairomone dose originating from *Anax imperator* larvae, adult *Triturus alpestris* and adult *Notonecta glauca*. Circle and square symbols indicate mean ± 1 SE of 7 replicate tubs. Triangle symbols shows results from 21 replicates of the water addition (control) treatment. *Anax imperator* and *Notonecta glauca* points are slightly offset for illustrative purposes. Lines represent the fitted model with the highest Akaike weight (*w*) (Table 1). The upper panel shows the proportion of visible tadpoles and the lower panel the proportion active of those observed. The vertical panels show the responses in three different tadpole ages: left panels 19 days old, middle panels 36 days old and right panel 48 days old. The values on the Y axis represent residuals after correcting for population differences.

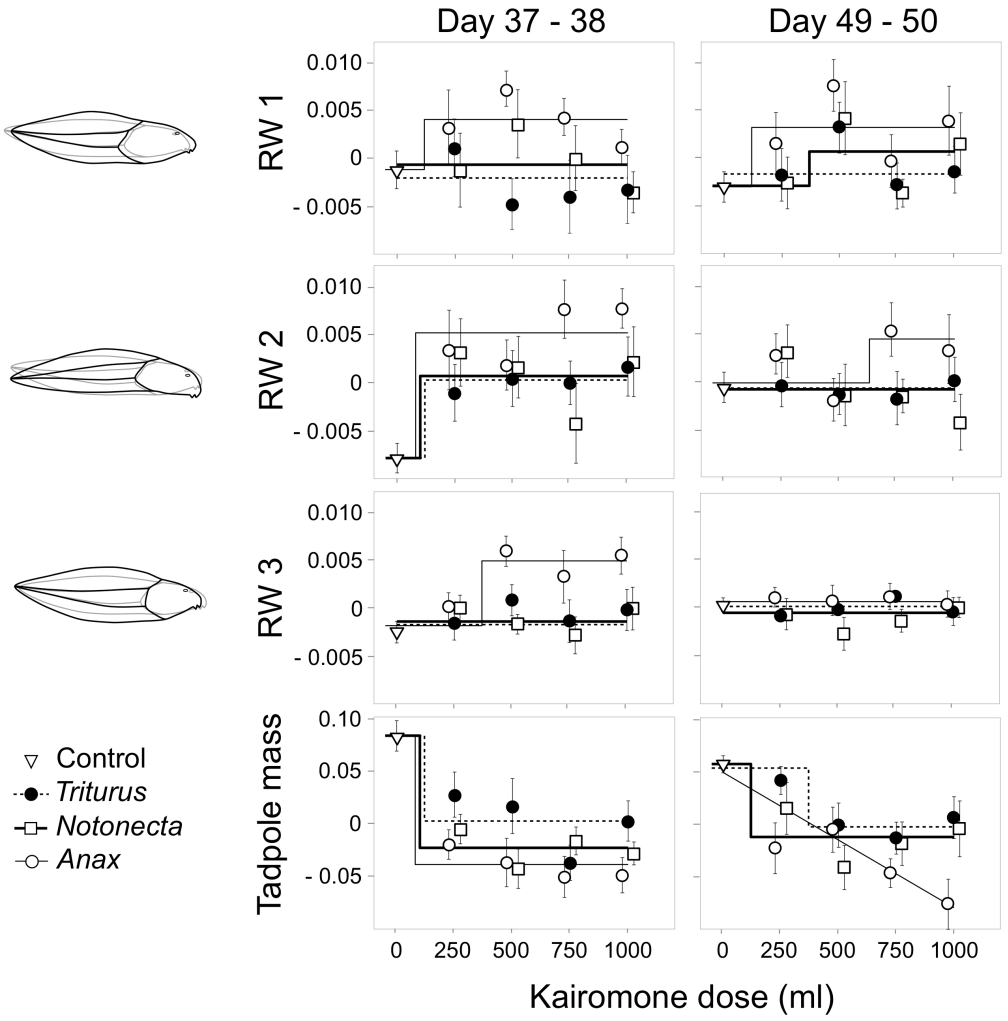

Figure 2. Morphological responses in shape and mass of *Rana temporaria* tadpoles to the increase of kairomone dose in ml of *Anax imperator* larvae, *Triturus alpestris* and *Notonecta glauca*. Circle and square symbols indicate mean ± 1 SE of 7 replicate tubs. Triangle symbols shows results from 21 replicates of the water addition (control) treatment. *Anax imperator* and *Notonecta glauca* points are slightly offset for illustrative purposes. Lines represent the model with the highest Akaike weight (*w*) (see Table. 1). The vertical panels show the responses in two different tadpole ages. The first three horizontal panels show the responses in the three different relative warps (RW) with major eigenvalues. The tadpole outline in black represents the individuals with high scores on the RW. The lower horizontal panels show the tadpole mass response. The values on the Y axis represent the residuals of the indicated trait name vs. population.

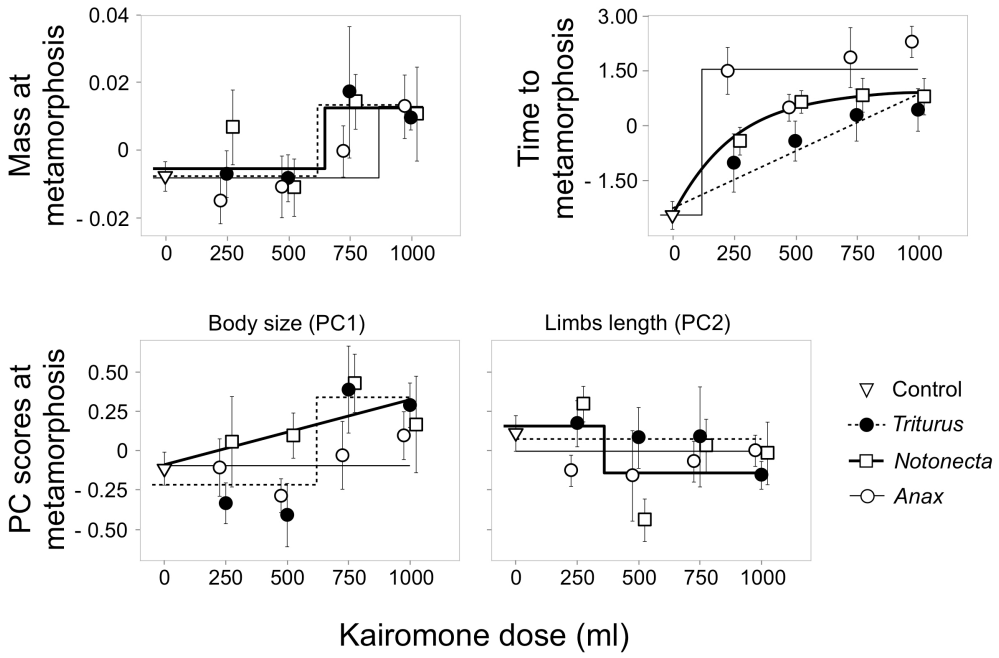

Figure 3. Responses at metamorphosis of *Rana temporaria* tadpoles to the increase of kairomone dose in ml of *Anax imperator* larvae, *Triturus alpestris* and *Notonecta glauca*. Circle and square symbols indicate mean ± 1 SE of 7 replicate tubs. Triangle symbols shows results from 21 replicates of the water addition (control) treatment. *Anax imperator* and *Notonecta glauca* points are slightly offset for illustrative purposes. Lines represent the model with the highest Akaike weight (*w*) (see Table. 1). The upper left panel shows the response in mass at metamorphosis, the upper right panel the response in time to metamorphosis, and the lower panels the metamorph morphology: body size (left) and limbs length (right). The values on the Y axis represent the residuals of the indicated trait name vs. population.

CHAPTER 2

EFFECTS OF COMBINED PREDATORS ON TADPOLE PHENOTYPES: A REPLACEMENT SERIES APPROACH TO MULTIPLE TRAIT RESPONSES

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Abstract

The important role of predator diversity in the functioning of ecosystems has motivated researchers to evaluate how prey respond to different predator species. However, focusing only on how prey respond to separated predator species precludes the detection of the effects that could arise when different predators occur in combination. I examined how tadpoles of *Rana temporaria* responded to variation in predation risk, signaled by variation in the relative amounts of kairomones in three kind of pairwise combinations of predator species. Overall, I found that tadpoles exhibit reduction in behaviour and in tadpole mass, increase in morphological shape traits, in the time to metamorphosis, and in mass at metamorphosis. However, depending on the trait evaluated and the predator combination, I found that tadpoles use hierarchical responses, synergic responses or a general response. These results show that the effect of changing the relative amount of predator kairomone on tadpole responses is reflected in different ways for different traits. The results also suggest that, depending on the differences in predator tactics and on the relative probabilities of encountering each predator, tadpoles can use different decision rules to produce responses.

Introduction

Predator induced defenses are responses to variation in environmental risk (Tollrian and Harvell 1999). In nature, such environmental risk is represented often by multiple predator species (Sih et al. 1998). When the predators in these assemblages have different predatory tactics and each induce specific prey responses, in multiple-predator situations the prey must adjust their responses in proportion to the probability of encounter with each. Thus, depending on whether the predators have independent effects, it is expected that the prey responses increase or decrease proportionally to the relative abundance of each predator species (i.e., linear aggregated effects). However, alternatively, interactive effects can arise, either because the predators interact directly among themselves or because an adaptive response to one predator renders the prey more vulnerable to another (Lima 2002). In both of these cases, the result may be reduction or enhancement in the prey response relative to the expected response (i.e., non-linear effects) (DeWitt and Langerhans 2003). Then it is expected that the prey reduce or enhance their response gradually as the relative density of each individual predator in the assemblage increases or decreases. Despite many studies evaluating response to multiple predator species (Relyea 2003), little has been done to evaluate how variation of the relative predator density in assemblages affects prey phenotypic responses, even though predator variation is the common situation that prey experience in natural communities.

The ability of prey to perceive changes in predation risk is a key component in the regulation and maintenance of predator-induced defenses (Tollrian and Harvell 1999, Lima and Steury 2005). In aquatic systems, water-borne chemical kairomones are one important cue signaling risk. Extensive study of inducible defenses using anuran tadpoles and predator kairomones has documented how variability in the kairomone type and amount produces variation in prey response. For example, studies using separately caged predators demonstrated that tadpoles can produce predator-specific responses, suggesting prey discriminatory abilities to differentiate predator kairomones (Relyea 2001, Storfer and White 2004, Teplitsky et al. 2004). Moreover, gradually increasing amounts of kairomone from different predator species, signaling an increase of specific predator risk, result in specific dosage-response curves in behavioural, morphological and life history traits (see Chapter 1). These results illustrate that kairomones in aquatic environments represent important public information that aquatic prey use to assess quantitatively and qualitatively the environmental risk. Given that prey can discriminate among predators, one can predict that prey may identify and weigh the components in combined predator conditions and adjust their responses depending on the relative proportion of the specific signal in the total information available.

In multiple predator conditions, the predators commonly act simultaneously. Therefore, to test extensively how prey respond to combined predators, in the study of the multiple predator effects, it is necessary to include experimental treatments in which predator species are present alone, as well as

in combination with other species (Relyea 2003). The goal is to identify whether the interactions between predators cause reduction or enhancement in the prey response. A commonly employed method to examine interference between two components is the standard replacement series, or substitutive, design (Jolliffe 2000). This approach evaluates whether the intra- and inter-specific components of interference are in balance. The standard replacement series comprises a set of treatments in which the proportions of each component vary from 0 to 100%, holding the total density constant. The results are represented by so-called yield response diagrams that illustrate how the response varies as one component is substituted in the mixture for a second component. When the components act independently and combine linearly, the yield response is a straight line connecting the two pure conditions (Sih et al. 1998). Therefore a linear model is the frame of reference to identify whether the response enhance or reduce as the components in the mixture change. Although some studies evaluating combined predator effects have used replacement series, their designs have included only one 50:50 combined condition. Such reduced number of conditions limits an extensive interpretation of the effects of the predator density and diversity on the prey response. To overcome these potential difficulties, multiple mixtures of water-born kairomones can be created, generating multiple intermediate points between the pure conditions. Thus, the use of kairomones, instead of predators themselves, in the treatments can reveal how prey phenotypes vary with changes in the relative abundance of predator species in assemblages.

In wetlands of middle Europe, larvae of *Anax imperator* dragonflies and adults of *Notonecta glauca* backswimmers and *Triturus alpestris* newts occur simultaneously in different relative densities and prey heavily on tadpoles (Van Buskirk and Arioli. 2005). In *Rana temporaria* tadpoles, specific dosage-responses to variation in the kairomones produced by these three predator species have been measured experimentally (Chapter 1). Therefore, this tadpole species and the kairomones of these predators offer the possibility to evaluate, using the replacement series design, how prey respond to variation in the relative density of predator species in combined predator configurations. My goal then was to investigate whether a gradual change in the relative amount of predator kairomone in pairwise predator combinations results in yield responses with gradual tadpole response reduction or gradual tadpole response enhancement, or whether the effect of the predators combines linearly. I measured the behaviour, morphology and metamorphic performance responses of *R. temporaria* tadpoles to the addition of water, pure kairomones and kairomone combinations in three different proportions for the three possible pairwise predator combinations.

Methods

I conducted the experiment in artificial ponds, raising tadpoles of *Rana temporaria* under different levels of apparent predation risk created by daily addition of kairomones obtained from the locally-common predators *A. imperator*

larvae, *T. alpestris* adults and *N. glauca* adults. I used pure kairomone treatments in high dose (1000 ml), and mixed kairomone treatments in which I added combined kairomones from different predators in different doses. Here I summarize the experimental design, protocols, and methods for measuring phenotype. For further details see Chapter 1.

The study included 13 predator treatments, assigned in a completely randomized design to 105 experimental units. Every pond received 1000 ml of water each day. In the predator-free control treatment, the water was aged tap water. In the other 12 treatments, the water was conditioned with predator kairomones. Each of the three predator species had one treatment in which I added pure predator-conditioned water; for each pairwise predator combination three treatments combining kairomones of the two predators in the ratios 3:1, 2:2, and 1:3. There were seven replicates for each kairomone treatment and 21 replicates for the predator-free control. The experimental units were plastic tubs (0.28 m^2, 80 L), filled with tap water, and stocked with 50 g of leaf litter, 2 g of rabbit food, and concentrated zooplankton to establish mesocosms that simulate natural pond conditions. I covered the mesocosms with a cloth lid to prevent colonization by aquatic predators or other amphibians. The experiment began on 23 March 2008, when I introduced 14 tadpoles (stage 23 – 25, Gosner 1960; 15.7 mg) to every mesocosm. The tadpoles came from clutches of *Rana temporaria* collected on 13 and 14 March in seven different ponds, distributed over an area of ~120 Km^2 in northern Switzerland. The experiment ended when the last froglet emerged.

The diluted kairomones were produced by keeping predators individually for 24 hours after feeding in 1L plastic boxes. Every predator was fed daily within the box with ~300 mg of *R. temporaria* tadpoles (4-6 tadpoles). On the next day, I collected the water contained in the boxes, refilled them with aged tap water, and fed the predators again. The water containing kairomones was diluted to a total volume of 48L and was distributed to the mesocosms according to the assigned treatment.

Phenotypic responses to predator kairomones

A total of 18 phenotypic responses to variation in apparent predation risk were obtained by sampling behaviour and morphology of tadpoles and measuring metamorphic features at stage 45 (Gosner 1960; tail resorption complete). Behaviour was measured when the tadpoles were 19, 36 and 48 days old, by visiting the mesocosms 6 - 7 times per day and counting the number of tadpoles that were visible and the number that were active. The proportion visible was the number of individuals observed divided by the initial number in the tank (14 tadpoles; survival range at the end of the experiment: 12 to 14 individuals). The proportion of tadpoles active was the number of tadpoles swimming or feeding divided by the number of visible tadpoles.

Tadpole size and shape were measured when tadpoles were 37-38 and 49-50 days old on a subsample of five tadpoles from each mesocosm. They were

weighed and photographed in lateral view within a small water-filled Plexiglas chamber using a digital camera Fuji S602Z ®. I used image J software to digitize the 31 landmarks defined in Appendix A, and used geometric morphometric methods to describe variation in shape and size. This analysis was performed using generalized least squares Procrustes superimposition (GLS) and the IMP software written by H.D. Sheets (http://www3.canisius.edu/~sheets/moremorph.html). The partial warps obtained were subject to principal component analysis, which produced 58 PC axes (shape components, termed relative warps - RW). The first three RWs, which accumulated 74.3% of the variance in shape, were retained for later analyses.

At forelimb emergence (stage 42) the tadpoles were removed from the mesocosms and kept in translucent plastic boxes. The time to metamorphosis was recorded at complete tail resorption (stage 45) and, for metamorph morphology, a haphazard subsample of five of the individuals in stage 45 from each mesocosm was photographed and weighed. I made six morphological measurements on the images of froglets: body length (snout-vent length), head width, width of the upper hind leg, width of the lower hind leg, upper hind leg length and lower hind leg length (Appendix B.1). On these measurements I performed a principal component analysis, and retained the two first components to represent body size (PC1: 84.71% of the variance) and relative limb length (PC2: 9.90%) (Appendix B.2).

Statistical analysis

To evaluate the yield responses of the tadpoles to the kairomone treatments, I performed contrast analyses and model selection on the residuals of the traits after controlling for differences among replicates (ponds of origin). For response variables in which ANOVAs detected significant effects of treatment ($P < 0.05$; six levels per pairwise predator combination), I compared specific treatments using planned contrasts. For each pairwise predator combination, the first contrast compared the five kairomone treatments against the predator-free control. The second contrast compared differences among the two pure kairomone treatments, and a third contrast compared the mixed kairomone treatments against the midpoint of the two pure kairomone treatments (Appendix C.1). If the first contrast was significant and the second and third contrasts both had P values > 0.10 (a stringent condition), I concluded that all five predator kairomone treatments produce the same effect on the trait relative to the control. Thus, this group of traits, by default, was classified as cases in which predator effects combine linearly. On the other hand, if either the second or third contrast had $P < 0.1$ (a generous condition), I subjected the trait to further analysis of the replacement series. Using the small-sample version of Akaike Information Criteria (AICc; Burnham and Anderson 2002), I used Akaike weights (w) to decide among three alternative models relating the trait to the gradual replacement of predator kairomones along the series (4:0, 3:1, 2:2, 1:3, and 0:4; Appendix D). The first model was a linear model that reflects an additive

combination of signals from the two predators ($y = \beta_0 + \beta_1$*[predator 2 dose]). The second alternative was a second order model that indicates a response dominated by one of the two predators ($y = \beta_0 + \beta_1$*[predator 2 dose] + β_2*[predator 2 dose]^2). The final alternative was a third order model used to identify cases in which tadpoles responded more strongly to one predator than the other only at some mixtures. Data processing and statistical analysis were performed using R version 2.9.1.

Results

The effect of treatment was significant in 11 of the 18 traits for the pairwise combination *Anax* – *Triturus*, 13 for *Anax* – *Notonecta* and 11 for *Triturus* – *Notonecta* (ANOVA, $P < 0.05$; Appendix C.2). In all cases, the contrast between the predator-free control and the five predator kairomone treatments was significant (Appendix C.2). These contrasts reflected a reduction in activity and visibility, a reduction in tadpole mass, an increase in RW shape scores, increase in the time to metamorphosis, and an increase in mass at metamorphosis in predator treatments relative to the control. Among these cases, there was no variation among the five predator mixtures (i.e., $P > 0.10$ for the second and third contrasts) for 4 of the 11 traits for the pairwise combination *Anax* – *Triturus*, 4 of 13 for the combination *Anax* – *Notonecta* and 8 of 11 for the combination *Triturus* – *Notonecta*. For these 16 traits these results indicate that the predator effects combine linearly.

Table 1 shows the model selection results for traits in which there was variation among predator treatments. I found for the *Anax* – *Triturus* combination that four trait responses were best described by the linear model, two cases by the second order model and only one case by the third order model. Data from the *Anax* – *Notonecta* combination best supported the second order model in many cases, the linear model in one case, and the third order model in three cases. The *Triturus* – *Notonecta* combination had only three cases: two were best supported by the second order model and one by the third order model (Table 1). Behavioral traits often showed a second-order curve, indicating that the response to kairomone mixtures was usually dominated by one of the two predators. Behaviour was best explained by the third order model only for *Anax* – *Notonecta*, because activity and visibility in all three mixture treatments was approximately intermediate between the two pure conditions. The behaviour response data best supported the linear model only for the *Anax* – *Triturus* pair and occurred in visibility at day 36 (Fig. 1).

In tadpole shape, I found $P < 0.10$ for contrast 2 or contrast 3 only for *Anax* – *Triturus* and *Anax* – *Notonecta* pairwise cases, and this occurred only at day 37-38 (Appendix C.2). For the first combination, RW2 was best explained by the linear model and RW3 by the third order model. For the second pair, the RW1 and RW3 responses best supported the second order model. Visual inspection of the nonlinear responses in Fig. 2 suggests for the two pairwise

combinations a hierarchical response in RW3, with dominant effect of *Anax,* and for the *Anax* – *Notonecta* pairwise that the RW1 score was higher when predator kairomones were in combination than when kairomones were pure.

Tadpole life history traits showed for the three predator combinations nonlinear responses in tadpole mass in the early larval stage and linear response for the *Anax* – *Triturus* and *Anax* – *Notonecta* pairwise combinations in the late larval stage; for the time to metamorphosis the response was linear for the *Anax* – *Triturus* pairwise and nonlinear for the *Anax* – *Notonecta* pairwise (Table 1). At day 37 – 38, mass was best explained by the second order model for *Anax* – *Triturus* and *Anax* – *Notonecta*, while the third order model was better supported for this trait in the *Triturus* – *Notonecta* pair (Fig. 3). The nonlinear response in time to metamorphosis for the *Anax* – *Notonecta* pair best supported the second order model, in which tadpoles reacted to mixed kairomones as if they were pure *Anax* (Fig. 3).

Discussion

In this study, I determined how tadpoles responded to variation in the relative amounts of kairomones in pairwise combinations of predator species, extending early studies that evaluate how prey respond to combined predators. The results agree with many previous studies in showing that numerous behavioral, morphological, and life history traits respond to predators. I observed that tadpoles exhibit reduction in visibility and activity to all the predation

conditions, but they do not always produce morphological responses. Also, there were cases in which a general response was deployed for all the predator conditions, and there were other cases in which different alternative responses were observed. These alternative responses were identified in the predator pairs of species nearly half the time, except for the *Triturus* – *Notonecta* pair, for which the response to *Notonecta* was stronger for just three of 18 traits. These results show that the effect of changing the relative amount of predator kairomone on tadpole responses is reflected in different ways for different traits, indicating specific-trait decision-making. Furthermore, I conclude that tadpoles can use kairomones to estimate the relative abundance of predators in the environment and use this information to adjust their phenotypes.

Prey often use inducible defenses to diminish the impacts of predation (Tollrian and Harvell 1999). Reduction of activity is a widespread predator-induced defensive behaviour in tadpoles, which improve their survival (Skelly 1994) by interrupting the predator cycle at the early pre-contact stages (Jeschke 2006). On the other hand, morphological responses, specifically changes in tail shape of tadpoles, also predator-induced, reduce predator attack success at contact stages by improving swimming performance or causing predator distraction (Van Buskirk and Relyea 1998, Van Buskirk and McCollum 2000, Teplitsky et al. 2005). In the case of the interactions between the predators used here and tadpoles, the responses observed apparently may be adapted to the differences in the hunting mode, prey manipulability mode and spatial habitat of each predator (Van Buskirk 2001, also see Chapter 1). *Notonecta* is a visual

predator that uses the open water column, whereas *Anax* and *Triturus* move slowly through the vegetation or along the bottom searching for prey. The behavioural responses observed may be used by the tadpoles facing *Notonecta* to reduce the probability of detection, and by the tadpoles facing *Anax* or *Triturus* to reduce the probability of encounter. On the other hand, *Notonecta*, which is the smallest predator among the three, uses its front limbs to hold prey while it sucks its internal tissues; *Triturus* consume their prey by engulfing them in only one strike; and *Anax* hold the prey with their labial palps while using external mandibles to consume it. These differences in consumption mode impose more limits in prey manipulation to *Notonecta* and *Triturus* than to *Anax*. Thus, at contact stages of the predator cycle, due to differences in the predator manipulability, the morphological responses observed may be used by the tadpoles to reduce the predator's attack efficiency when the tadpoles cannot obtain protection in the size refuge. Because *Triturus* and *Notonecta* are more limited in prey manipulability than *Anax*, this strategy may be used mainly when tadpoles face *Anax* predators.

Early studies have shown that different predators may induce phenotypic responses in prey in the same direction or in opposite direction, and for both cases, prey may respond to the predators in combination by producing a response dominated by one of the predators involved or by producing an intermediate response (see review in Relyea 2003). However, because prey can produce fine tuned gradual responses as the density of predator increase (Van Buskirk and Arioli 2002, Schoeppner and Relyea 2008, 2009), when the relative

predator density vary in the combination, the above-mentioned responses may be identified by the yield responses observed in the replacement series diagrams. Thus, when predators in separated conditions produce different responses, non linear yield responses would indicate the dominance cases and linear yield response would indicate the intermediate responses. But if predators in separate conditions produce similar responses, a unique generalized response may be observed in which prey maintain the same level of response regardless of the specific conditions of the predator combination. In my study, this occurred in about 30% of the traits that I measured. However, cases of synergic response in the combination can be observed if prey responses in the combination are stronger than in the two predators separated conditions.

Responses dominated by one predator in a combination should arise when that predator is perceived as more dangerous than the others and the different predators select responses in the same direction but with different magnitude (Teplitsky et al. 2004, Bourdeau 2009). In my results, at early larval stages, it was observed that the three predators induce behavioural responses in the same direction but it appeared that *Notonecta* is perceived as more dangerous for the tadpoles. These differences in tadpole perception of predators were observed in dosage responses produced at the same developmental stage, where *Notonecta* induced threshold dosage responses while *Anax* and *Triturus* induced continuous dosage responses (see chapter 1). If the behavioural responses are adapted to the differences in the spatial habitat used by these predators, at early stages, the size refuge protection could be very low for the

tadpoles. Then, in the combined conditions, adopt the response to face *Anax* or *Triturus* would increase the tadpole vulnerability to *Notonecta*. Therefore, the tadpole behavioural responses at early stages must be one dominated by *Notonecta*, as observed here. But as tadpoles grow larger, they can obtain protection in the size refuge. Thus, at mid way in development, in contrast to the early stages, due to limitations in prey manipulability of *Triturus* and *Notonecta*, tadpoles may gain protection by size refuge against them but still be vulnerable to *Anax*. Therefore, it is likely that at mid stages tadpoles switch to consider *Anax* as the more dangerous predator. My results, showing strong relative responses in morphology and in visibility at mid developmental stage to *Anax*, support that tadpoles indeed change the perception of risk as they grow up, as has been suggested also in early studies (Van Buskirk 2001, Relyea 2003). Then, if the tail shape is adapted to reduce the predator's attack success when there is not size refuge protection, in combined conditions, tadpoles that base their protection only on a size refuge would increase the probability of attack success by *Anax*. Therefore, at mid stages in development, once the behavioural defenses are no longer effective, it may be better for tadpoles in multi-predator conditions to be constituted with a tail shape useful to evade *Anax*. This is what I observed in RW3 shape score in which the three predators induce tails with larger surfaces than in the predator-free condition; but in the combined conditions tadpoles responded similar to the response produced in the *Anax* condition.

Generalized responses may arise when the perception of risk imposed by different predators is equivalent, the predators in isolation induce similar

responses and facilitation among predators when they are in combination does not occur. Homogenization of the perception of risk of different predators can occur as the prey grow up, identified by constraints on the inducible defenses over ontogeny (Relyea 2005). At early developmental stages, when there is no size refuge, my results suggest that vulnerability to *Anax* and *Triturus* is similar. This could occur because the probability of encounter with the two is equivalent due to their similar spatial habitat use. At mid stages the perception of risk changes due to partial protection from a size refuge and vulnerability to *Triturus* and *Notonecta* becomes more similar. Finally, at late stages it is likely that a size refuge confers protection against all three predators. Thus, variation in vulnerability through development may explain why generalized defenses arise for some traits. I observed generalized behavioural responses for the *Anax*-*Triturus* combination at early stages, in the *Triturus*-*Notonecta* combination midway through development, and in all three pairwise combinations at late stages. For morphology, generalized responses were observed in the combinations of *Anax* – *Triturus* and *Triturus* – *Notonecta* at the two late stages. It cannot be argued that tadpoles are unable to differentiate the three species of predator, because results for other traits and data presented in chapter 1 illustrate that *R. temporaria* often exhibit different responses to different predators. Therefore, my results suggest that generalized defenses arise as an alternative strategy that prey sometimes deploy when they face multiple predators, rather than from an inability to assess or process detailed information about predation risk.

Soluk and Collins (1988) propose that synergic responses arise when positive interaction between predators occurs. For example, indirect facilitation among predators can mean that the total prey mortality rate is greater than the additive predicted mortality (Sih et al. 1998). Under these conditions, the response must be stronger than the one used in a dominance case. Synergic responses were observed only for the *Anax* – *Notonecta* combination and occurred in the behavioural responses and in the morphological response in the shape component RW1, at midway through development. This result suggests first that the limit in the tadpole behavioural responses and in the morphological responses is not expressed with the more dangerous predator. Second, the result indicates that at mid sizes these two predators may produce mutually facilitation in predation. This may occur because tadpoles avoiding areas where they encounter *Notonecta* become exposed to *Anax* and vice versa. When such kinds of conflicts arise, overestimating the risk would be the more convenient decision rule (Lima and Steury 2005). Thus, the outcome of this kind of risk perception may result in the increase of the magnitude of the responses when prey are in combined conditions. Therefore, my results suggest that tadpoles can overestimate the risk, but this decision rule depends on the ecological circumstance they are facing like the kind of predator assemblage.

Blumstein and Bouskila (1996) suggest that decision-making process arises from the stimulus-response stage that couples the assessment of risk information with the evaluation of the benefits and cost associated with all the alternative responses. Because the behavioural responses carry costs by

reducing foraging opportunities, and morphological responses carry costs by allocation of energy, traits like mass and time to metamorphosis can reveal the consequences of the decision making process in tadpoles for the tadpoles' energy budget (Relyea 2002). In my study, tadpole mass at mid developmental stages indicates that the responses used during early and middle development in the combined conditions have strong costs. Mass was strongly reduced in the combined conditions relative to the pure conditions. This result suggests that short term extra costs exist for tadpoles facing simultaneously more than one predator. However, long term costs in my study do not support Blumstein and Bouskila's (1996) argument. Tadpole mass was similar in the combined predator treatments late in development (linear yield response), and final mass measured in metamorphs was not affected or showed a generalized unique response. It seems that tadpoles have the opportunity to compensate for costs early in development by growing faster late in development, somehow recovering the energy allocated to the induced phenotypes produced at early stages (Van Buskirk and Saxer 2001).

This multi-trait approach allowed me to identify that the tadpoles can use different decision rules to produce responses that are adjusted to differences in predator hunting strategies and to the relative probabilities of encountering each predator. The data also suggest that tadpoles can shift such decision rules over ontogeny, which apparently has an adaptive basis. Thus, determining how prey respond to combined predation risk in which the relative density of predators in combined conditions varies has given new insights in the functional ecology and

evolution of predator induced phenotypes. Additionally, my results emphasize the importance of including predator diversity and density simultaneously in ecological models, to allow a better understanding of the dynamic of populations and community structure in natural complex systems.

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Tables

Table 1. Akaike weights (w) of the models used for model selection based on the small sample AICc for traits measured on tadpoles of *Rana temporaria* exposed to treatments in which the composition of the kairomones of pairwise predators was manipulated. The values of w are indicated for three pairwise predator combinations: a) *Anax imperator* larvae - *Triturus alpestris* adults, b) *Anax imperator* - *Notonecta glauca* adults and c) *Triturus alpestris* - *Notonecta glauca*. The traits listed are those in which the planned contrasts two or three indicated in the appendix C.2 had P values < 0.1. Except for the traits measured at metamorphosis, the tadpole age day when the measurement was obtained is indicated after the trait name. K is the number of estimable parameters in the model. The best fitting model is highlighted in boldface. The Akaike weights for all traits sum to 1. RW are relative warps obtained from geometric morphometric analysis (Fig. 2).

Trait	Model		
	Lineal	2nd order	3rd Order
K	1	2	2
a) *Anax - Triturus*			
Visibility - Day 36	**0.532**	0.161	0.307
Activity - Day 36	0.021	**0.745**	0.234
Tadpole mass - Day 37-38	0.027	**0.578**	0.395
Tadpole mass - Day 49-50	**0.601**	0.180	0.218
RW2 - Day 37-38	**0.462**	0.306	0.233
RW3 - Day 37-38	0.221	0.272	**0.507**
Time to metamorphosis	**0.554**	0.224	0.222
b) *Anax - Notonecta*			
Visibility - Day 19	0.001	0.199	**0.800**
Activity - Day 19	0.001	0.140	**0.860**
Visibility - Day 36	0.001	**0.930**	0.069
Activity - Day 36	0.001	0.041	**0.958**
Tadpole mass - Day 37-38	0.001	**0.895**	0.105
Tadpole mass - Day 49-50	**0.599**	0.187	0.214
RW1 - Day 37-38	0.000	**0.941**	0.059
RW3 - Day 37-38	0.010	**0.680**	0.310
Time to metamorphosis	0.088	**0.643**	0.269
b) *Triturus - Notonecta*			
Visibility - Day 19	0.030	**0.774**	0.195
Activity - Day 19	0.012	**0.738**	0.251
Tadpole mass - Day 37-38	0.001	0.119	**0.880**

Figures

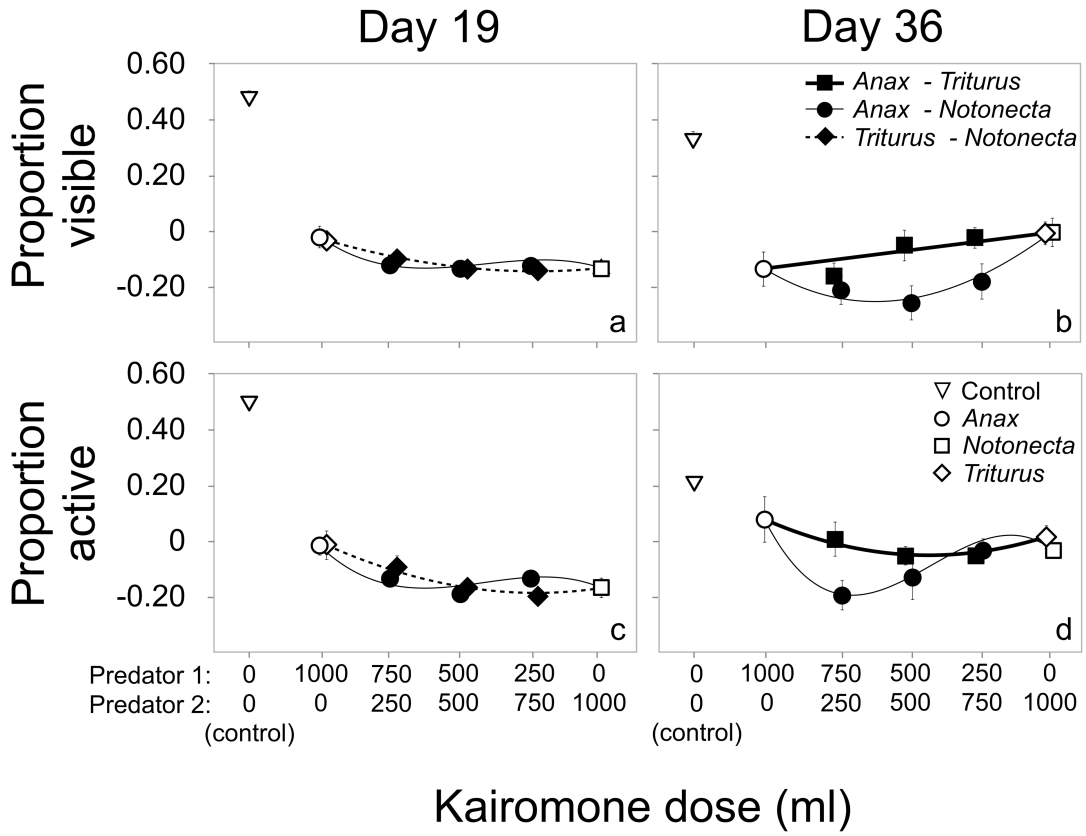

Figure 1. Behavioural responses of *Rana temporaria* tadpoles to the gradual replacement of predator kairomone amount in pairwise predator species. The first predator named in the pairwise combination corresponds to Predator 1 and the second to Predator 2: *i*) *Anax imperator* larvae - *Triturus alpestris* adults, *ii*) *Anax imperator* - *Notonecta glauca* adults and *iii*) *Triturus alpestris* - *Notonecta glauca*. Open black symbols indicate the mean response to pure kairomone doses ± 1 SE of 7 replicate tubs and the solid black symbols indicate the mean response to the mixed kairomone doses ± 1 SE of 7 replicate tubs. The triangle symbol shows the mean response to the water addition (control) ± 1 SE of 21 replicates, plotted as a reference to indicate the direction of response. Points for the *Anax imperator* - *Triturus alpestris* pair and *Triturus alpestris* - *Notonecta glauca* pair are offset 20 ml left and right respectively for illustrative purpose. The cases illustrated were those in which the planned contrasts two or three (appendix C.2) had P values < 0.1. Lines represent the fitted model with the highest Akaike weight (*w*) (see Table 1). Panels a and b show the proportion of visible tadpoles, and panels c and d the proportion active of those observed. The vertical panels show the responses in two different tadpole ages: a and c panels 19 days old and b and d panels 36 days old. Values on the Y axis represent residuals after correcting for pond differences.

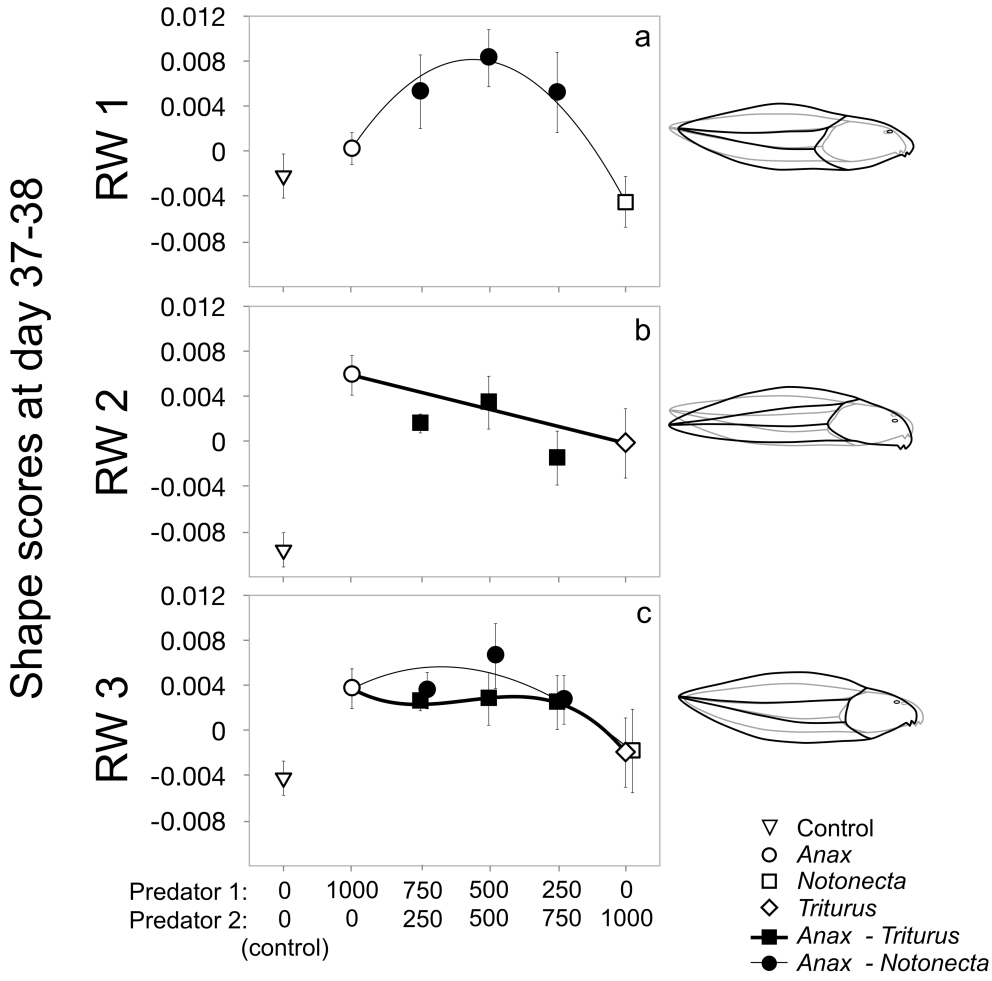

Figure 2. Morphological responses in shape of *Rana temporaria* tadpoles at age 37 - 38 to the gradual replacement of predator kairomone amount in pairwise predator species. The first predator named in the pairwise combination corresponds to Predator 1 and the second to Predator 2: *i*) *Anax imperator* larvae - *Triturus alpestris* adults, *ii*) *Anax imperator* larvae - *Notonecta glauca* adults and *iii*) *Triturus alpestris* adults - *Notonecta glauca* adults. Open black symbols indicate the mean response to pure kairomone doses ± 1 SE of 7 replicate tubs and the solid black symbols indicate the mean response to the mixed kairomone doses ± 1 SE of 7 replicate tubs. The triangle symbol shows the mean response to the water addition (control) ± 1 SE of 21 replicates, plotted as a reference to indicate the direction of response. *Anax imperator* - *Triturus alpestris* pairwise points are offset 20 ml left for illustrative purpose. The cases illustrated were those in which the planned contrasts two or three indicated in the appendix C.2 had P values < 0.1. Lines represent the fitted model with the highest Akaike weight (*w*) (see Table 1). The tadpole outline in black represents the individuals with high scores on the RW. Values on the Y axis represent residuals after correcting for pond differences.

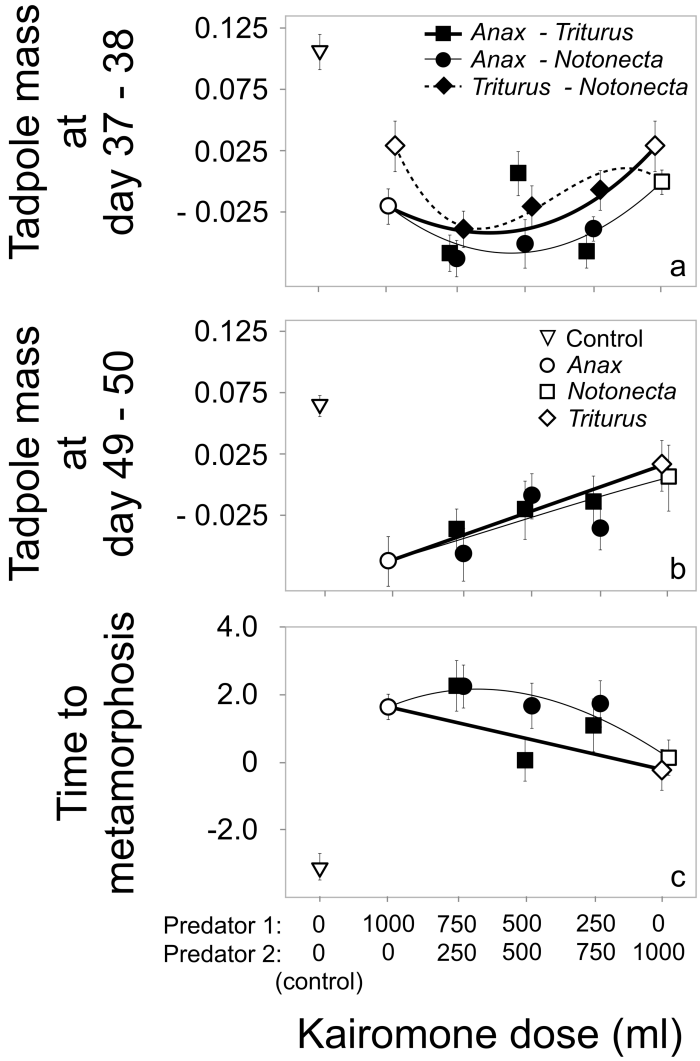

Figure 3. Tadpole mass at two ages (a and b) and time to metamorphosis (c) responses of *Rana temporaria* tadpoles to the gradual replacement of predator kairomone amount in pairwise predator species. The first predator named in the pairwise combination corresponds to Predator 1 and the second to Predator 2: *i*) *Anax imperator* larvae - *Triturus alpestris* adults, *ii*) *Anax imperator* larvae - *Notonecta glauca* adults and *iii*) *Triturus alpestris* adults - *Notonecta glauca* adults. Open black symbols indicate the mean response to pure kairomone doses ± 1 SE of 7 replicate tubs and the solid black symbols indicate the mean response to the mixed kairomone doses ± 1 SE of 7 replicate tubs. The triangle symbol shows the mean response to the water addition (control) ± 1 SE of 21 replicates, plotted as a reference to indicate the direction of response. *Anax imperator* - *Triturus alpestris* pairwise and *Triturus alpestris* - *Notonecta glauca* pairwise points are offset 20 ml left and right respectively for illustrative purpose. The cases illustrated were those in which the planned contrasts two or three indicated in the appendix C.2 had P values < 0.1. Lines represent the fitted model with the highest Akaike weight (*w*) (see Table 1). Values on the Y axis represent residuals after correcting for pond differences.

CHAPTER 3

MULTIPLE PREDATOR EFFECTS ON TADPOLE SURVIVAL

Oscar Javier Ramos Real

Abstract

Although the number of studies interested in the effects of predator diversity and trophic interactions on the ecosystems has increased in the recent years, they have focused mainly on identifying whether non-additive effects arise at interspecific level (emergent multiple predator effects). However, intraspecific effects may be also critical in the study of the structure and function of ecological systems. In predation trials experiments, using *Anax imperator*, *Triturus alpestris* and *Notonecta glauca* predators, I evaluated density dependent effects and multiple predator effects on prey survival by exposing tadpoles to conditions in which the type of predator and the number of individual predators foraging together varied. I used model selection to determine whether the increase in intraspecific density caused non-additive effects and I employed the additive and substitutive experimental designs to determine whether emergent multiple predator effects occurred. This study shows at the intraspecific level, that there were additive effects due to *Triturus* and *Notonecta* predators, whereas the predation rate of *Anax* was strongly reduced when the number of predator individuals increased (non-additive effects). On the other hand, the additive and the substitutive approaches demonstrated that the predator effects observed on tadpole survival in combined predator conditions were predicted from the effects observed when the predator species were in isolation, indicating that the three pairwise combinations produced additive effects on the tadpole survival. Thus, my results indicate that independent effects were prevalent in the predator systems evaluated here, with exception of the density dependent effects observed in *Anax* dragonflies.

Introduction

Predation is one of the most important factors influencing the structure and function of ecological systems (Lima 2002, DeWitt and Langerhans 2003). In nature, multiple predator species are often present together, and they may have different effects on the abundance, species diversity, morphology, behaviour and life history of their prey (Tollrian and Harvell 1999, Van Buskirk 2001, Relyea 2003, Teplitsky et al. 2004). Hence, each predator species may represent a specific functional unit with a distinct effect on the lower level in the food web. Thus, when multiple predators feed simultaneously on a common prey, the ecological function of the predator assemblage may depend on the predator diversity and on the variety of interactions that arise among the species included in the predator – prey system (Schmitz 2007). If the predators have independent effects, their impacts will combine additively. But if the predators are engaged in interactions their effect may not combine additively, producing overall risk reduction or risk enhancement; both of these are considered to be emergent multiple predator effects (MPE) (Sih et al. 1998).

Although ecological systems with multiple predators have been identified in the majority of food webs, most studies have examined predator effects using single predators in isolation. Such oversimplification has precluded the detection of the diversity and the density effects that could arise under multiple predator conditions (Sih et al. 1998, Relyea 2003). However, some recent studies have

examined the effects of predator species in combination. These studies have discovered that predation by multiple species often does not combine additively, and instead produces emergent impacts on the prey (i.e. non-additive effects) (Losey and Denno 1998, Nystrom et. al. 2001, Vance-Chalcraft et. al. 2004, Vance-Chalcraft and Soluk 2005, Griffen 2006). Sih et al. (1998) have suggested that the factors causing these non additive effects are competitive interference, intraguild predation among the predators or interaction modifications (i.e. indirect effects of predator or prey behavioural changes). These arguments are applied at the interspecific level, but the same ecological factors can also affect how conspecific predators foraging together impact the prey (Vance-Chalcraft et. al. 2004, Griffen 2006). In other words, risk reduction or risk enhancement may also occur when multiple individuals of the same species forage together. Therefore, to evaluate predation in multiple predator systems, one must examine non additivities at both levels: the intra and inter-specific levels.

Emergent MPEs are identified when the observed impacts on the prey in combinations of predators cannot be predicted from the impacts caused when the predators are in isolation. This approach uses a null model that assumes that the predators have independent effects and that their effects sum additively. Methodologically, two different designs have been used to examine emergent MPEs: the additive and the substitutive experimental designs (Griffen 2006, Schmitz 2007). The more common additive approach evaluates the collective net effect of all the predator species as predators are added to the system (Billick

and Case 1994), while the second, less frequently used, substitutive approach evaluates whether the predator species have substitutable effects on the prey (Jolliffe 2000). Because predator diversity and density covary using the additive approach, it is impossible to identify which of the two factors causes interactions, if they occur. On the other hand, the substitutive approach evaluates the effects of the interactions between species relative to the effects of interactions between conspecifics, while density is held constant. This approach identifies whether the strength of the effect of one species on the prey depends on whether other predator species are present. Because the two designs consider density and diversity in different ways, they address different questions. However, a better comprehension of emergent MPE can be obtained by using both designs in tandem, inferring how the predator diversity and density impact prey in multiple predator systems, including the effects of the increase of intraspecific density.

Odonate larvae, newts and backswimmers are aquatic predators that have different hunting strategies and impose different mortality rates on tadpoles (Van Buskirk and Arioli 2005). As these predators co-occur simultaneously in ponds, it is possible that interactions occur among them. Therefore, emergent MPEs could be observed on the tadpoles exposed to combinations of these predators. In this paper, using these predators and the additive and the substitutive experimental designs, I evaluated the predation effect on the survival in *Rana temporaria* tadpoles in multiple predator conditions. I determined whether there are non additive effects due to the presence of multiple conspecific individuals of the

same species and due to the presence of multiple predator species. Specifically, I used the three predator species *Anax imperator* dragonfly larvae, *Notonecta glauca* backswimmer adults, and *Triturus alpestris* newt adults, under all three possible pairwise combinations, to evaluate (1) whether the increase in intraspecific density of each species caused nonadditive effects on tadpole survival and (2) whether there were emergent multiple predator effects (risk reduction or risk enhancement) when tadpoles faced combined predator species.

Methods

In predation trial experiments, I exposed tadpoles of the frog *Rana temporaria* to treatments that varied in the type of predator and the number of individual predators foraging together to evaluate the impacts of predator diversity and predator density on survival. To evaluate this, I used the three pairwise systems obtained from combining *Anax imperator*, *Triturus alpestris* and *Notonecta glauca* predators. For each combination, I used a full complement of predator treatments, including a 2-by-2 factorial design with the presence and absence of one predator individual of each predator species, along with two additional treatments, which had for each predator species the presence of two conspecific predator individuals (Fig 1). The experiments were conducted in eight one-day trials between 14 April and 7 May 2009, using plastic tubs (0.28 m^2, 80 L) located outdoors in a field on the University of Zurich campus. In each trial, five replicates of each predator treatment and three of the predator-free treatment were randomly assigned to 48 tubs. After eight repetitions, this

produced a total of 40 replicates for the predator treatments and 24 replicates for the predator-free treatment. Tubs were filled 24 hours before starting the trial and stocked with two packets of ribbons made of plastic mesh (each with 4 ribbons 40 cm x 4 cm) to provide habitat structure. One packet floated and the other, attached to a 120 g stone, lay on the bottom. Two hours after being filled with water, each tub received 12 tadpoles of *R. temporaria* (60 - 80 mg). Predators were introduced at 8:00 the next morning, and removed 9 hours later at 17:00, at which point survivors were counted. The predators and surviving tadpoles were released in the pond of origin, and every subsequent trial used a new set of animals.

Statistical analysis

Tadpole survival rate per hour was estimated as the slope m in the function $\ln(S_t) = \ln(S_0) + mt$, where S_t was the number of surviving tadpoles counted at the end of the day-trial, $S_0 = 12$ (the number of tadpoles introduced at the beginning) and $t = 9$ (the total duration of the predation trials). Because there was variation in survival among days ($F_{7,376} = 6.629$, $P < 0.001$), I performed the subsequent analyses on the residuals of m vs. day-trial (hereafter referred to as tadpole survival).

For each predator species, I determined whether the increase in intraspecific density caused nonadditive effects on tadpole survival by comparing three alternative models relating survival to the number of predators. I used a

linear model to identify whether the conspecific predators had independent linear aggregated effects, a second order model to identify whether nonlinear effects arose, and I used a null model, including only the intercept, to identify the cases in which the predator species did not affect tadpole survival. Akaike weights (*w*) obtained from the small-sample Akaike Information Criteria (AICc) were used to identify the best model supported by the tadpole survival data from this set of models (Burnham and Anderson 2002).

In each pairwise predator system, I determined whether emergent multiple predator effects occurred by contrasting the observed effect to the expected effects, using both the additive and substitutive approaches. Under the additive approach, I identified an emergent effect of combining predator species using the significance of the interaction term in a two-way ANOVA, with the two predator species as main effects having two levels: predator presence and absence (Billick and Case 1994). Under the substitutive approach, I evaluated whether the effects due to the interspecific interactions were as strong as that due to intraspecific interaction (Jolliffe 2000), testing whether the effect caused by the predators together is significantly different from the mean of the effects caused by each predator species when alone at the same total density. To do that, I analyzed the effect of the predator treatments with an ANOVA followed by a planned contrast, which evaluated whether the observed effect of the predator combination was different from the average of the effects observed in the

conditions with two conspecific individual predators. Data processing, statistical analyses and model fitting were performed using R version 2.9.1.

Results

Notonecta was the most dangerous predator, followed by *Anax* and *Triturus* (Fig 2). In analyses of dose-response curves within the three predators separately, the model linearly relating tadpole survival rate to predator density was best supported by the data for *Notonecta* and *Triturus*, and the quadratic model was best supported for *Anax* (Table 1). The rate at which individual *Anax* killed tadpoles was about half as great when two dragonflies were present in the tub as when only one was present (Fig. 2).

Tadpole survival observed in the two-predator combinations did not differ from that expected based on the additive or substitutive approaches. I found nonsignificant two-way interaction under the additive approach analysis for the three predator combinations (Table 2). Under the substitutive approach, for the combination *Anax* – *Triturus* there were no differences among the three predator treatments (ANOVA, P = 0.547, Table 3). For the *Anax* – *Notonecta* and *Triturus* – *Notonecta* combinations the contrasts were not significant (Table 3). This is clearly visible in Fig. 2 for the *Anax* – *Notonecta* and *Triturus* – *Notonecta* pairwise combinations: hourly survival rates lie almost exactly halfway between survival rates in the corresponding treatments with two conspecific predators.

Thus, the predators had independent effects when they were in combination and when multiple individuals of *Triturus* or *Notonecta* were present. Instead, when the density of *Anax* increased there was an intraspecific non-additive effect with risk reduction.

Discussion

This study using both additive and substitutive experimental approaches, demonstrates that pairwise combinations of the three predator species do not produce emergent multiple predator effects on the survival of *Rana temporaria* tadpoles. Indeed, the predator effects observed on tadpole survival in combined predator conditions were predicted by both approaches from the effects observed when the predator species were in isolation. These results suggest that hunting or foraging rates of the predator species did not change when they were in pairwise combinations, indicating also that they have independent effects on tadpole survival. At the intraspecific level, there were additive effects when the density of *Triturus* and *Notonecta* increased, but non additive effects when the density of *Anax* increased. While the predation rate of the first two species showed a linear relationship with the number of predator individuals, the predation rate of *Anax* was strongly reduced when the number of predator individuals increased. Thus, my results indicate that independent effects were prevalent in the predator systems evaluated here, with exception of the density dependent effects observed in *Anax* dragonflies.

Different conclusions can be drawn about the functional role of a predator in a multiple predator species assemblage as a consequence of the way in which the predator diversity and the predator density are manipulated (Sih et al. 1998, Griffen 2006). In fact, in most of the studies in which both approaches have been used simultaneously, the conclusions drawn from the additive approach are different from those drawn from the substitutive approach (Vance-Chalcraft and Soluk 2005, Griffen 2006, and see reexamined data in Schmitz 2007). My study differs from these in that the two approaches led to the same conclusion for the three predator systems evaluated. On one side, finding non significant interaction term under the additive design indicates that the effects of the predator species can be predicted from the effects in the isolated condition (Billick and Case 1994). Further, even though density and diversity covary and their effects can not be disentangled under this approach, my results indicate with certainty that the predators have independent effects. On the other hand the results obtained from the substitutive approach indicate that the level of intraspecific interference was about as strong as the level of interspecific interference. These results suggest that the predation rate of each species was not affected when a conspecific predator was replaced by a predator of another species, also indicating the independent effects of the predators. Together, these results show that neither risk reduction nor risk enhancement occurred among the predators. These results corroborate previous results using tadpoles, in which additive effects were observed under the additive approach (Van Buskirk 1988), and extend the

conclusions of other predator – prey systems in which additive effects have been observed among predators in pairwise combination (Sokol-Hessner and Schmitz 2002, Finke and Denno 2005, Griswold and Lounibos 2006). Further, while discrepancies have been found in studies that use both methods, in this study there was strong evidence supporting that neither density nor diversity affect the ecological function of the predators when they are in combined conditions.

Sih et al. (1998) suggest that the main mechanisms causing emergent MPEs (risk reduction or risk enhancement) are intraguild predation and interaction modifications such as conflict in the prey responses to different predator species or predator behavioural changes. Thus, the lack of emergent MPEs would be an indicator that these mechanisms are not taking place in the predator assemblages evaluated. In this study, for the three predator systems evaluated, the results suggest that these underlying factors did not occur or were too small to be statistically detected. It is evident that intraguild predation did not occur in this experiment because there were no missing predators at the end of the trials. Also, many studies suggest that conflicts in the responses may arise when prey face multiple predators (Lima 2002, Relyea 2003, Teplitsky et al. 2004). For example, prey behavioural defenses to one predator can put them at risk when facing other kinds of predators. In contrast, some animals can use generalized defenses against different predators. When this occurs, the generalized defense should be observed in simple predation conditions as well as in conditions in which different predators are in combination. Because my

experiment used naive tadpoles without any previous experience with predators, the development of morphological defenses was unlikely. Thus, behavioural defenses are the most likely defensive strategies during this study. Therefore, because I did not find emergent MPEs, if the tadpoles used a defense, it is likely that they were using a generalized behavioural strategy for all the predator conditions. This is plausible because the same kind of behavioural responses, hiding behaviour and reduction of activity, were induced on the *R. temporaria* tadpoles by the three predators used in this experiment (see Chapter 1). Thus, although I only have evidence that intraguild predation did not occur, the fact that the emergent MPEs were not observed suggests that tadpoles may be using a generalized defense and/or that behavioural changes in the predators did not occur. However, to better support these arguments, future experiments using combined predators should evaluate the behavioural changes of prey and predators to identify their influence on the predation rate.

Non additivities at the intraspecific level have received much less attention than non additivities at the interspecific level. In part, this is a result of the way in which the density and richness of predators have been manipulated in experiments evaluating multiple predator effects. In this study, the simultaneous use of both additive and substitutive designs gave an opportunity to evaluate how the increase in density within species affects the predation rate of the three predator species. My results indicate that density dependent effects do not occur for *Triturus* and *Notonecta* predators, whereas risk reduction was observed when

the density of *Anax* increased. This suggests that the mechanisms producing non additivities do not occur for *Triturus* and *Notonecta* species, but one or more of these mechanisms must be occurring for *Anax*. Although cannibalism between Odonata has been reported (Van Buskirk 1989), I did not observe missing *Anax* at the end of the predation trials. However, individuals of this predator species may change their behaviour to reduce the probability of cannibalism by reducing activity when conspecifics are present. If this occurs, their foraging effort could be reduced, affecting their predation rate on the prey. This suggests that risk reduction observed when two *Anax* individuals were together may be due to behavioural changes in the predators. An alternative explanation is that changes in prey behaviour occurred in response to increasing predator density. Therefore, predation rate may decrease if the prey intensify or shift their behaviour when the predator density increases. In chapter 1 I observed that an increase of kairomones, representing increase of predation risk, reduced the activity of tadpoles (see also Van Buskirk and Arioli 2002, Schoeppner and Relyea 2008). Thus, because cannibalism was not observed, it is possible that both the prey and predator behavioural changes affect the predation rate of *Anax* when their density increases.

These results indicate that the three species can be treated as single independent functional units in models that evaluate prey population dynamics in multiple predator systems. Further, the complementary design gave the opportunity to observe the density dependent effects of the three species. This

study is one step towards understanding how the complexities behind sources of risk affect prey population dynamics (Lima 2002, DeWitt and Langerhans 2003). And it highlights the importance of incorporating the dynamics of the predator diversity and the effects of intra and interspecific interference in the study of the predator-prey interactions.

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Tables

Table 1. Akaike weights of the models used for model selection based on the small sample Akaike Information Criteria (AICc) for survival of *Rana temporaria* tadpoles when exposed to variation in the number of individuals of three different predator species: *Anax imperator* larvae, *Triturus alpestris* adults and *Notonecta glauca* adults. K is the number of estimable parameters in the model. The best fitting model is highlighted in boldface. The Akaike weights always sum 1.

	Model		
	Intercept	Linear	2nd order
K	2	3	4
Anax	0.001	0.173	**0.826**
Triturus	0.006	**0.700**	0.294
Notonecta	0.000	**0.720**	0.280

Table 2. Results of ANOVAs used to compare the observed predation in pairwise combinations (a,b and c) of three predator species (*Anax imperator*, *Triturus alpestris* and *Notonecta glauca*) to the null expectation of independent predator effects, based on the additive experimental design (see Fig. 1). Significant P values are highlighted in boldface.

Source	df	SS	F	P
(a) *Anax – Triturus*				
Anax	1	0.024	22.524	**< 0.001**
Triturus	1	0.004	3.300	0.071
*Anax * Triturus*	1	0.002	2.303	0.131
Residuals	140	0.150		
(b) *Anax – Notonecta*				
Anax	1	0.013	4.279	**0.040**
Notonecta	1	0.089	28.846	**< 0.001**
*Anax * Notonecta*	1	0.003	1.093	0.298
Residuals	140	0.434		
(c) *Triturus – Notonecta*				
Triturus	1	0.007	3.242	0.074
Notonecta	1	0.146	64.307	**< 0.001**
*Triturus * Notonecta*	1	0.001	0.087	0.769
Residuals	140	0.318		

Table 3. Results of ANOVA and planned contrasts used to compare the observed predation in pairwise combinations (a,b and c) of three predator species (*Anax imperator*, *Triturus alpestris* and *Notonecta glauca*) to the null expectation of independent predator effects, based on the substitutive experimental design (see Fig. 1). Significant P values are highlighted in boldface. NA is indicated for the case in which the planned contrast was not performed, based on the ANOVA result.

ANOVA					Planned contrast			
Source	df	SS	F	P	Contrast		t	P
(a) *Anax* – *Triturus*								
Predator	2	0.002	0.607	0.547	$\frac{2Ax + 2Tr}{2}$	vs. AxTr	NA	NA
Residual	117	0.205						
(b) *Anax* – *Notonecta*								
Predator	2	0.105	12.214	**< 0.001**	$\frac{2Ax + 2Nt}{2}$	vs. AxNt	0.280	0.780
Residual	117	0.502						
(c) *Triturus* – *Notonecta*								
Predator	2	0.137	19.689	**< 0.001**	$\frac{2Tr + 2Nt}{2}$	vs. TrNt	1.341	0.183
Residual	117	0.408						

Figures

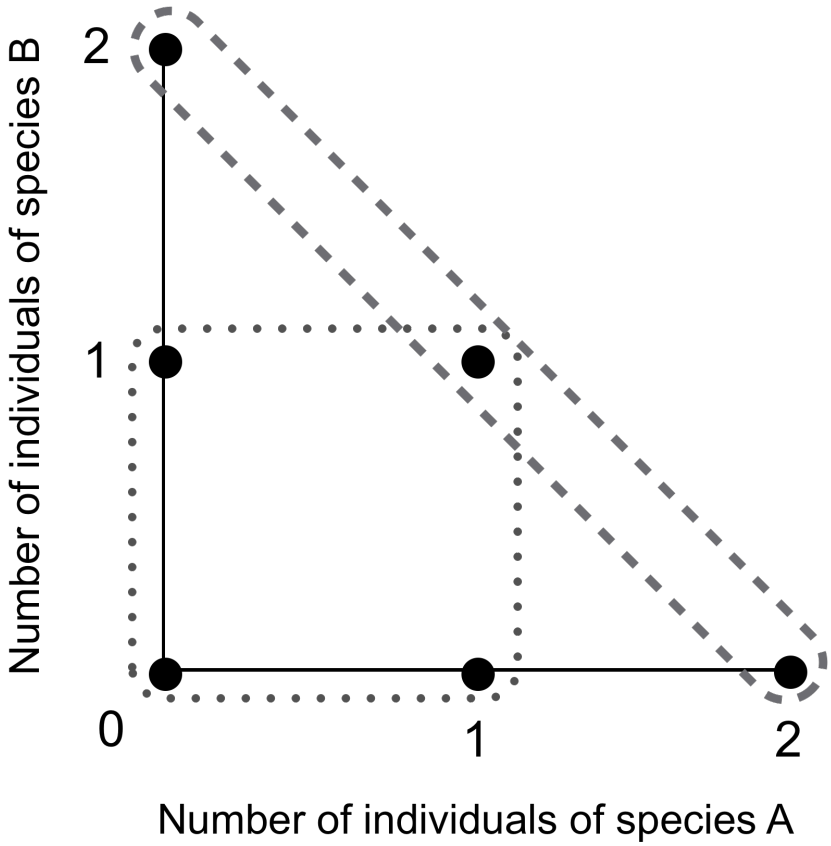


Figure 1. Design of the experiment to evaluate emergent multiple predator effects on mortality of *Rana temporaria* tadpoles caused by three kinds of predators (*Anax imperator* larvae, adult *Triturus alpestris* and adult *Notonecta glauca*). The experimental configuration shown here was repeated for each pairwise combination. A and B represent the two predator species and the black circles indicate the number of individuals of each species used in the 6 treatments. The dotted line illustrates the two-by-two factorial design and the dashed line the replacement series design.

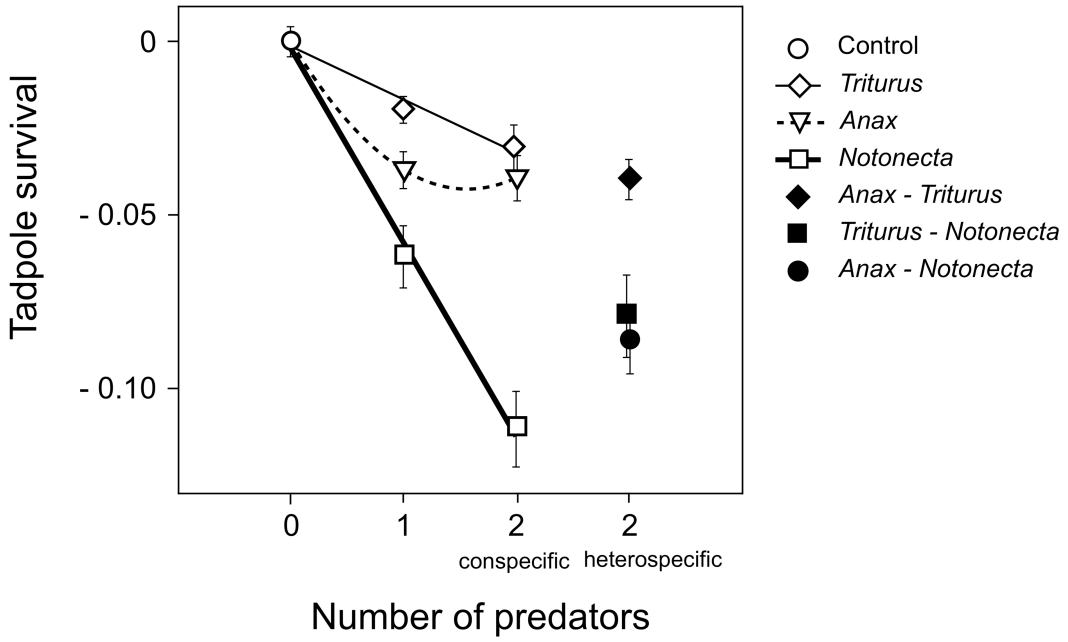
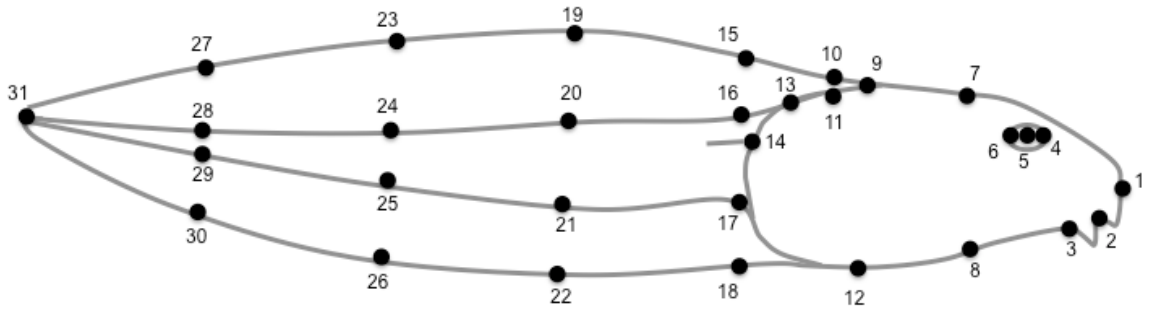


Figure 2. Survival of *Rana temporaria* tadpoles when exposed to different numbers and combinations of *Anax imperator* larvae, adult *Triturus alpestris* and adult *Notonecta glauca*. Symbols are means ± 1 SE. The open circle represents the predator-free control (24 replicates). Other open symbols are the single-species predator treatments (40 replicates). Filled symbols indicate the three hetero-specific predator treatments (40 replicates). Lines represent the fitted model with the highest Akaike weight (*w*) (Table 1). Symbols that overlap are offset intentionally for illustrative purposes. The values on the Y axis represent residuals after correcting for day-trial differences.

Appendix A

Landmarks on the side view of the *Rana temporaria* tadpoles.



1 Most anterior point on the nose

2 Center of the partially-opened mouth when viewed from the side

3 Junction of the posterior edge of the lower labium and the body wall

4 Anterior edge of the iris on a horizontal line extending through the center of the eye

5 Center of the pupil

6 Posterior edge of the iris

7 Upper edge of the head/body at the anterior gut margin

8 Lower edge of the head/body at the anterior gut margin

9 Point at which the dorsal tail fin attaches to the top of the head/body

10 Dorsal edge of tail fin at 2/3rds of the distance between #1 and #17

11 Dorsal edge of the head/body at 2/3rds the distance between #1 and #17

12 Ventral edge of the body at 2/3rds the distance between #1 and #17

13 Point where upper edge of the tail muscle meets head/body

14 Point where the notochord (identified from the pattern of myotomes) meets the head/body

15 Upper edge of the tail fin projected perpendicular to the line #17 - #31 at #17

16 Top of tail muscle point projected perpendicular to the line #17 - #31 at #17

17 Point where the bottom edge of the tail muscle meets the head/body

18 Lower edge of the tail fin projected perpendicular to the line #17 - #31 at #17

19 Upper edge of the tail fin at 1/4th the distance between #17 and #31

20 Top of tail muscle at 1/4th the distance between #17 and #31

21 Bottom of tail muscle at 1/4th the distance between #17 and #31

22 Ventral edge of the tail fin at 1/4th the distance between #17 and #31

23 Upper edge of the tail fin at halfway between #17 and #31

24 Top of tail muscle at halfway between #17 and #31

25 Bottom of tail muscle at halfway between #17 and #31

26 Ventral edge of the tail fin at halfway between #17 and #31

27 Upper edge of the tail fin at 3/4th the distance between #17 and #31

28 Top of tail muscle at 3/4th the distance between #17 and #31

29 Bottom of tail muscle at 3/4th the distance between #17 and #31

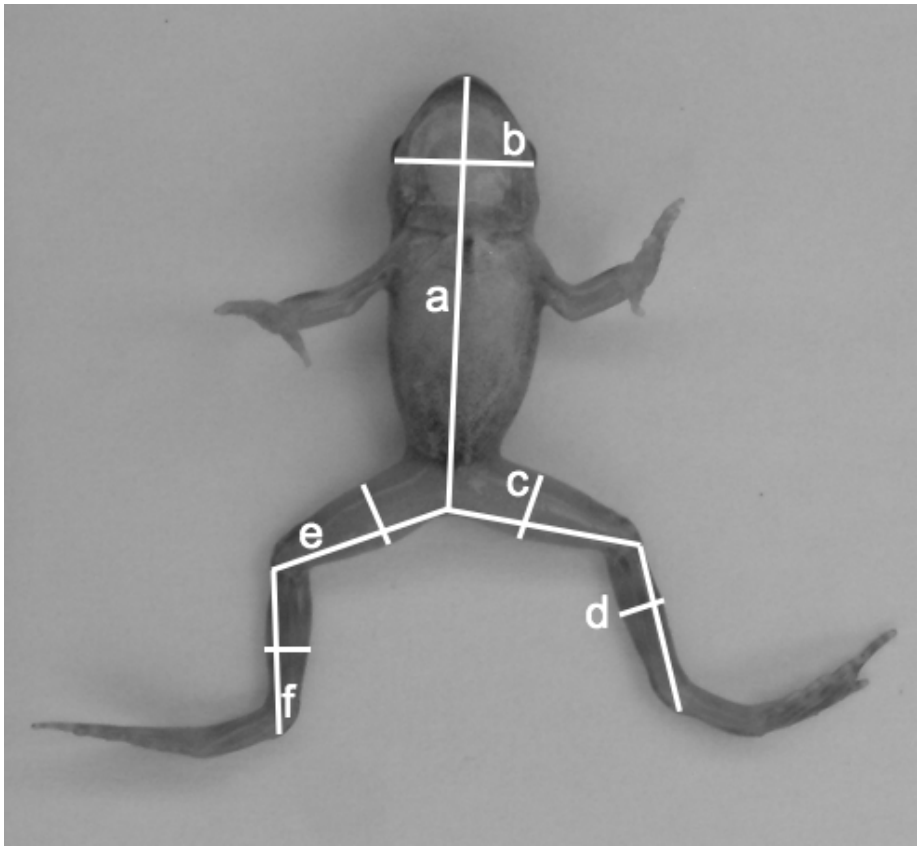
30 Ventral edge of the tail fin at 3/4th the distance between #17 and #31

31 Tip of tail fin

Appendix B

Appendix B.1

Body parts used for digital measurements of morphology at Gosner stage 45 of *Rana temporaria* metamorphs: (a) body length (snout-vent length), (b) head width, (c) width of the upper hind leg, (d) width of the lower hind leg, (e) upper hind leg length and (f) lower hind leg length.



Appendix B.2

Results of principal component analyses on the covariance matrix of six morphological traits measured on *Rana temporaria* metamorphs using varimax rotation. The values are coefficients of the first two eigenvectors, representing body size (PC1) and limb length (PC2). The measurements are defined in Appendix B.1. For paired structures, the analysis was performed on averages of the left and right measurements.

Morphometric variable	PC1 (Body size)	PC2 (Limbs length)
a. body length (snout-vent length)	0.930	0.365
b. head width	0.736	0.497
c. width of the upper hind leg	0.619	0.577
d. width of the lower hind leg	0.577	0.545
e. upper hind leg length	0.365	0.902
f. lower hind leg length	0.535	0.770
% of variance	84.71	9.90

Appendix C

Appendix C.1.

Planned contrasts evaluated in trait responses of *Rana temporaria* tadpoles that had significant effect in the predator treatment (ANOVA, $P < 0.05$, appendix C.2). Solid and open symbols indicate the treatments lumped together in the contrast.

Treatment	Contrast 1	Contrast 2	Contrast 3
Control Water	●		
Predator 1 P1 (1000 ml)	○	●	●
Predator 2 P2 (1000 ml)	○	○	●
Mixture 1 P1 (250 ml) - P2 (750 ml)	○		○
Mixture 2 P1 (500 ml) - P2 (500 ml)	○		○
Mixture3 P1 (750 ml) - P2 (250 ml)	○		○

Appendix C.2

ANOVA of the predator treatment effect on the *Rana temporaria* tadpole responses and planned contrasts performed between predator treatments. The analyses were performed for three pairwise predator combinations: a) *Anax imperator* larvae - *Triturus alpestris* adults, b) *Anax imperator* larvae - *Notonecta glauca* adults and c) *Triturus alpestris* adults - *Notonecta glauca* adults. Contrast 1 was the contrast between the predator-free control and the kairomone treatments; Contrast 2 was the contrast between the two pure kairomone treatments in the pairwise combination; and Contrast 3 was the contrast between the two pure kairomone treatments and the mixed kairomone treatments (see Appendix C.1). The P values < 0.05 in the ANOVA and in the Contrast 1, and the P values < 0.10 in the Contrast 2 and Contrast 3 are highlighted in boldface. NA is indicated for the cases in which the contrast analysis was not performed (ANOVA, P > 0.05).

Trait	ANOVA		Contrast 1		Contrast 2		Contrast 3		Residual std. error	R^2
	F	P	t	P	t	P	t	P		
a) *Anax - Triturus*										
Visibility - Day 19	88.655	**<0.001**	20.975	**<0.001**	0.253	0.801	1.288	0.204	0.092	0.899
Activity - Day 19	77.094	**<0.001**	19.504	**<0.001**	-0.058	0.954	0.892	0.377	0.099	0.885
Visibility - Day 36	26.153	**<0.001**	11.102	**<0.001**	-1.801	**0.078**	0.143	0.887	0.133	0.723
Activity - Day 36	9.212	**<0.001**	6.368	**<0.001**	0.950	0.347	1.877	**0.066**	0.122	0.480
Visibility - Day 48	19.528	**<0.001**	9.837	**<0.001**	-0.659	0.513	-0.024	0.981	0.101	0.661
Activity - Day 48	14.624	**<0.001**	8.237	**<0.001**	-0.615	0.541	-0.396	0.694	0.112	0.594
Tadpole mass - Day 37-38	17.573	**<0.001**	8.559	**<0.001**	-1.716	**0.092**	2.209	**0.032**	0.056	0.637
Tadpole mass - Day 49-50	10.152	**<0.001**	6.426	**<0.001**	-2.953	**0.005**	0.037	0.971	0.052	0.504
RW1 - Day 37-38	0.400	0.846	NA	NA	NA	NA	NA	NA	NA	NA
RW1 - Day 49-50	2.348	0.054	NA	NA	NA	NA	NA	NA	NA	NA
RW2 - Day 37-38	9.926	**<0.001**	-6.607	**<0.001**	1.793	**0.079**	0.766	0.447	0.006	0.498
RW2 - Day 49-50	0.369	0.868	NA	NA	NA	NA	NA	NA	NA	NA
RW3 - Day 37-38	4.555	**0.002**	-4.224	**<0.001**	2.002	**0.051**	-0.956	0.344	0.005	0.313
RW3 - Day 49-50	1.267	0.293	NA	NA	NA	NA	NA	NA	NA	NA
Mass at metamorphosis	1.929	0.106	NA	NA	NA	NA	NA	NA	NA	NA
Body size at metam (PC1)	1.678	0.157	NA	NA	NA	NA	NA	NA	NA	NA
Limbs length (PC2)	0.680	0.640	NA	NA	NA	NA	NA	NA	NA	NA
Time to metamorphosis	16.339	**<0.001**	-8.463	**<0.001**	1.998	**0.051**	-0.721	0.474	1.736	0.620

Appendix C.2. Continued

Trait	ANOVA		Contrast 1		Contrast 2		Contrast 3		Residual std. error	R^2
	F	P	t	P	t	P	t	P		
b) *Anax - Notonecta*										
Visibility - Day 19	142.870	**<0.001**	26.540	**<0.001**	2.613	**0.012**	1.752	**0.086**	0.080	0.935
Activity - Day 19	201.470	**<0.001**	31.361	**<0.001**	3.872	**<0.001**	2.471	**0.017**	0.073	0.953
Visibility - Day 36	39.959	**<0.001**	13.578	**<0.001**	-1.879	**0.066**	3.269	**0.002**	0.130	0.800
Activity - Day 36	15.214	**<0.001**	7.647	**<0.001**	1.580	0.120	3.126	**0.003**	0.131	0.603
Visibility - Day 48	27.281	**<0.001**	11.579	**<0.001**	-0.396	0.694	1.244	0.219	0.096	0.732
Activity - Day 48	13.130	**<0.001**	7.796	**<0.001**	-1.201	0.235	-0.524	0.603	0.109	0.568
Tadpole mass - Day 37-38	21.369	**<0.001**	10.010	**<0.001**	-0.723	0.473	2.307	**0.025**	0.053	0.681
Tadpole mass - Day 49-50	11.460	**<0.001**	6.905	**<0.001**	-2.533	**0.014**	0.327	0.745	0.053	0.534
RW1 - Day 37-38	4.058	**0.004**	-2.560	**0.014**	1.233	0.223	-3.375	**0.001**	0.007	0.289
RW1 - Day 49-50	4.095	**0.003**	-3.738	**<0.001**	0.556	0.581	-1.580	0.120	0.008	0.291
RW2 - Day 37-38	11.402	**<0.001**	-7.109	**<0.001**	1.530	0.132	-0.394	0.695	0.007	0.533
RW2 - Day 49-50	0.883	0.500	NA	NA	NA	NA	NA	NA	NA	NA
RW3 - Day 37-38	7.448	**<0.001**	-5.193	**<0.001**	2.056	**0.045**	-1.933	**0.059**	0.005	0.427
RW3 - Day 49-50	0.117	0.988	NA	NA	NA	NA	NA	NA	NA	NA
Mass at metamorphosis	2.053	0.087	NA	NA	NA	NA	NA	NA	NA	NA
Body size at metam (PC1)	1.413	0.236	NA	NA	NA	NA	NA	NA	NA	NA
Limbs length (PC2)	0.502	0.773	NA	NA	NA	NA	NA	NA	NA	NA
Time to metamorphosis	19.889	**<0.001**	-9.670	**<0.001**	1.629	0.110	-1.679	**0.099**	1.713	0.665
c) *Triturus - Notonecta*										
Visibility - Day 19	163.580	**<0.001**	28.425	**<0.001**	2.481	**0.017**	1.573	0.122	0.075	0.942
Activity - Day 19	165.150	**<0.001**	28.313	**<0.001**	3.571	**0.001**	2.276	**0.027**	0.080	0.943
Visibility - Day 36	21.873	**<0.001**	10.341	**<0.001**	-0.048	0.962	1.524	0.134	0.133	0.686
Activity - Day 36	19.330	**<0.001**	9.635	**<0.001**	0.968	0.338	1.368	0.177	0.093	0.659
Visibility - Day 48	24.068	**<0.001**	10.955	**<0.001**	0.323	0.748	0.121	0.905	0.089	0.707
Activity - Day 48	11.725	**<0.001**	7.249	**<0.001**	-0.614	0.542	-1.208	0.233	0.101	0.540
Tadpole mass - Day 37-38	15.494	**<0.001**	8.376	**<0.001**	1.115	0.270	2.144	**0.037**	0.051	0.608
Tadpole mass - Day 49-50	5.117	**0.001**	4.923	**<0.001**	0.407	0.685	0.975	0.334	0.048	0.339
RW1 - Day 37-38	0.357	0.875	NA	NA	NA	NA	NA	NA	NA	NA
RW1 - Day 49-50	0.820	0.541	NA	NA	NA	NA	NA	NA	NA	NA
RW2 - Day 37-38	4.383	**0.002**	-4.475	**<0.001**	-0.122	0.903	0.163	0.871	0.008	0.305
RW2 - Day 49-50	0.469	0.797	NA	NA	NA	NA	NA	NA	NA	NA
RW3 - Day 37-38	2.314	0.057	NA	NA	NA	NA	NA	NA	NA	NA
RW3 - Day 49-50	0.160	0.976	NA	NA	NA	NA	NA	NA	NA	NA
Mass at metamorphosis	2.677	**0.032**	-3.006	**0.004**	-0.100	0.920	0.019	0.985	0.021	0.211
Body size at metam (PC1)	1.950	0.103	NA	NA	NA	NA	NA	NA	NA	NA
Limbs length (PC2)	1.419	0.234	NA	NA	NA	NA	NA	NA	NA	NA
Time to metamorphosis	15.355	**<0.001**	-8.202	**<0.001**	-0.472	0.639	-0.681	0.499	1.437	0.606

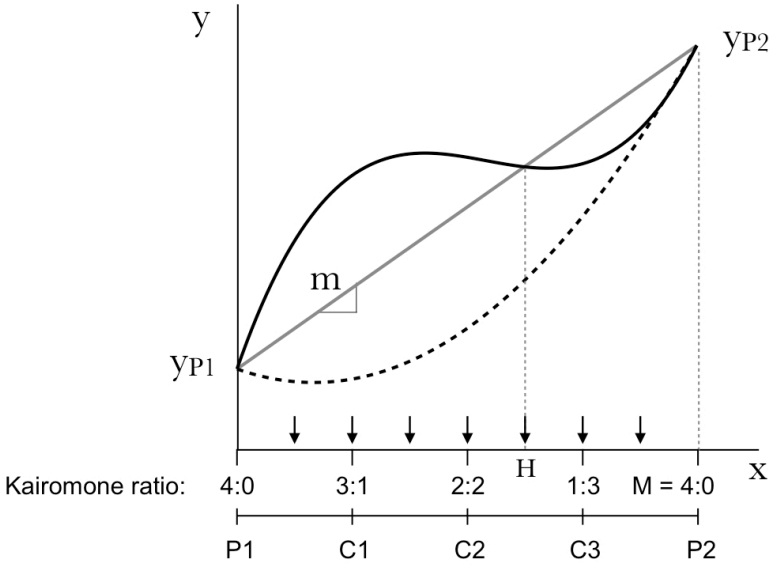
Appendix D

Models defined for model selection based on the small sample Akaike Information Criteria (AICc). The models related the tadpole response to the gradual replacement of predator kairomones, evaluated in five different kairomone ratios (figure D.1). Epsilon (ε) is the error parameter.

Model	Estimated parameters (K)	
$y = \alpha + mx$	ε	K = 1
$y = \alpha + mx + \beta\left(x^2 - Mx\right)$	β and ε	K = 2
$y = \alpha + mx + \beta\left[\left(\frac{HM^2 - H^2M}{M - H}\right)x - \left(\frac{M^2 - H^2}{M - H}\right)x^2 + x^3\right]$	β and ε	K = 2

Note: M, alpha, m and H were fixed parameters in the model. The first three were the base to evaluate the substitutability. M was assigned to Predator 2 (1000 ml), alpha was the response to the pure treatment Predator 1 (Y_{P1}) and m was the slope of the line connecting the two pure treatments (figure D.1). For the 3rd order model a preliminary selection was performed among models in which H was located in seven equidistant positions between the two pure-predator treatments (see figure D.1). The value of H that produced the best model was included in the final model selection.

Figure D.1. Illustrative representation of the models used to fit tadpole responses to mixed and pure predator kairomone treatments. In solid black the third order model, in dashed black the second order model and in gray the linear model. Y_{P1} indicate the response to the pure dose of Predator 1 (1000 ml) and Y_{P2} the response to the pure dose of Predator 2 (1000 ml). The arrows indicate the seven equidistant positions predefined for H; in the figure one case is illustrated.



$$m = \frac{(y_{P2} - y_{P1})}{M}$$

P1: Predator 1 (1000 ml) - Predator 2 (0 ml)
C1: Predator 1 (750 ml) - Predator 2 (250 ml)
C2: Predator 1 (500 ml) - Predator 2 (500 ml)
C3: Predator 1 (250 ml) - Predator 2 (750 ml)
P2: Predator 1 (0 ml) - Predator 2 (1000 ml)

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